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ON THE FOOD OF NUDIBRANCHS

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Apart from certain cases in which still little is known with certainty, our knowledge of the food of nudibranchs is very meagre. Likewise, the question of the food of many of them and its relation to their occasional disappearance by death or migration seem to be matters of confusion among many workers (Hecht, 1895; Chambers, 1934). At Al-Ghardaqa, where it has been possible to observe these animals in their natural habitat and to rear them in laboratory aquaria, advantage was taken to elucidate many points of interest in this regard.

Material and Methods

The species studied were collected from the tidal and subtidal coral patches in the immediate vicinity of the Marine Biological Station at Al-Ghardaqa on the Red Sea (27° 13′ N. Lat., and 34° 45′ E. Long.). The preferred habitat of these animals was found to be on dead coral skeleton overgrown by weeds. It is probable that these animals cannot stand strong light and high temperature for a long time, tending thus to hide by day in crevices. Hence, the best catches were those carried out in the early morning or about dusk. The animals were either killed and examined immediately after collection or reared in laboratory aquaria. The latter had a continuous current of sea water run through fine jets strong enough to form a cloud of air bubbles, thus ensuring perfect aeration and continuous movement of the water. In some aquaria pieces of living stony corals, as for instance *Favia, Goniopora, Coeloria,* as well as many forms of anemones, sponges, and algae, were introduced. Others were deprived of all kinds of food other than the planktonic content of the running sea water. A third group was supplied with sea water which had previously been passed through a cotton filter so as to keep back even planktonic organisms contained in the sea water. The last aquaria had been filled at the beginning of the experiment with filtered sea water so that the animals in them had nothing that they might feed on.

Results

Examination of the gut contents—or the wastes discharged at intervals through the anal aperture—of many of those species kept in the laboratory revealed exceedingly little, as they often contain practically nothing. On the other hand the in-

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vestigation of the gut contents of some chromodorids—killed immediately after collection—does reveal the presence of diatoms and zooxanthellae. In one case of *Chromodoris pulchella,* the oesophagus was found to contain two polychaete postlarvae on their way to ingestion. Careful investigation of its stomach inclusions indicated the presence of small fragments, evidently of the same polychaete species, in various stages of digestion. Included in the gut, also, were a copepod and a considerable amount of calcareous spicules of sponges, as well as very few algal filaments. It seems probable that this individual was feeding on sponges when it ingested some of these polychaete larvae. In many specimens of *Chromodoris quadricolor, Chromodoris annulata* and *Chromodoris ghardaqana,* the gut contents showed nothing but unidentifiable fragments.

Two species of the nudibranch genus *Phyllodesmium* were found to be specific for browsing on *Xenia* and *Heteroxenia* at Al-Ghardaqa (Gohar, 1940; Gohar and Aboul-Ela, 1957b). In sections through their cerata, the latter were found to contain a great amount of zooxanthellae in their hepatic caeca. It would be well to mention here that these zooxanthellae are certainly derived from their alcyonarian food. Thus, when a specimen was taken from a colony of *Heteroxenia fuscescens* which had been kept for more than ten days in a dark room aquarium, it looked as pale as the colony. In sections the cerata were found practically devoid of zooxanthellae.

In another nudibranch—namely, *Dermatobranchus striatus*—the only substratum on which it has been observed is *Clavularia hamra.* Thus, several individuals of this nudibranch were picked out of freshly collected colonies of *Clavularia* immediately after their introduction into laboratory aquaria. The fact that it is always observed on this substratum has led to the conclusion that it is specific for browsing on *Clavularia.* It may be well to refer to the observations of Gohar (1948) in which he points out that *Pleuroleura striata* (a synonym of *Dermatobranchus striatus*) browses on the softer parts of that alcyonarian. In sections, nematocysts were observed in its skin and zooxanthellae in various stages of intracellular and extracellular digestion in its gut. Furthermore, the same author states (p. 22) that, "Both the nematocysts and zooxanthellae are evidently derived from the eaten-up polyps." Although Bergh (1905) gave a detailed description of this nudibranch, no mention was made of its substratum, its food or the possession of nematocysts and zooxanthellae.

By comparing the gut contents of many specimens with the food materials of their habitat, it is found difficult to tell what is actually made use of. To investigate the preferred food taken by some of the nudibranchs under investigation, feeding experiments, using the broth of various animals and plants, were conducted. The most interesting results were those obtained by using sea urchins' gonads, fish, various anemones, crustacean ova, sponges, alcyonarians and algae. Applying the broth of sea urchins' gonads with a pipette to the front end of *Chromodoris quadricolor* or *Chromodoris annulata,* the animal makes progressive movements towards the stimulus and works its oral apparatus continuously, sucking in the broth. When solid pieces of the same food were used, the radula—protruded through the dilated oral tube—is firmly applied to the food material. On retraction, the radular teeth puncture the superficial cells, the fluid contents of which are sucked into the buccal cavity by the muscular contraction and expansion of the buccal mass.

Similar results were obtained by using some algae and crustacean eggs, while the broth of anemones and fish particles—prepared in the same way—were refused.

Other nudibranchs like *Hexabranchus sanguineus* invariably refused all food of animal source but responded well to that of plant origin like pounded algae.

Under field conditions, large colonies of *Discodoris erythraeensis,* as well as the tectibranch *Berthellina citrina,* were found to disappear by the end of the breeding season from places where I used to find many of them, together with their spawn ribbons (Gohar and Aboul-Ela, 1957a; 1959). The same took place in the case of *Trevelyana bicolor,* of which twenty-three specimens and twenty-one of its egg-ribbons were collected on June 20, 1952, from a large colony at Qad El-Tair on the shore of Shadwan Island. A month later, a long search to find even a single specimen in this locality proved fruitless. In the laboratory, the animals supplied with most of the food they might have in their natural habitat could be kept in good health for several months. Several of them deposited their egg-ribbons more than twice during the experiment. Although some of the animals supplied with plankton, only, began to show signs of ill-health, others survived quite a long time and deposited their spawn ribbons. The individuals deprived of all kinds of food could hardly survive after the first week. The disappearance of the above colonies should not lead one, therefore, to assume that the animals die after oviposition but—being found accompanied by a large number of their egg-ribbons—it is most probable that they come near shore to spawn and migrate at the end of the breeding season to places where more favourable conditions prevail for the adults.

Discussion

The above observations demonstrate that many of the species investigated show a definite choice of their diet. Thus, while some forms like *Phyllodesmium xeniae* and *Dermatobranchus striatus* subsist mainly on animal matter, which in this case are the alcyonarians, many of the chromodorids here examined subsist for one part of their food on animal matter and for another part on plant diet. A third group like *Hexabranchus sanguineus* is entirely herbivorous, depending more or less completely on vegetable matter for food supply.

The absence of any appreciable amount of food material in the gut of many of the specimens investigated indicates that nudibranchs—as an adaptation to their sluggish life—have acquired the capacity of living on a minimal amount of food. Thus it may be that these animals under normal conditions do not, as a rule, take large amounts of food at one time but browse slowly, and that digestion proceeds as rapidly as ingestion resulting in very little, if any, food material in the guts of even freshly collected specimens.

The relation of food of nudibranchs to their occasional disappearance by death or migration is equally significant. According to one school of naturalists the disappearance of large colonies of nudibranchs can be explained as a result of a reduction in the food supply in a given locality, leading to their death or migration. Another school is of the opinion that this disappearance is primarily due to their immediate death after spawning. In the systematic literature, there is a widespread acceptance of supposed migratory phenomenon succeeding spawning and related processes.

Chambers (1934) recorded the sudden disappearance of *Embletonia fuscata* Gould, two weeks after the collection of several hundred individuals. His efforts to find even one specimen in the same locality were in vain, and this led him to assume the migration of the entire colony. In the opinion of this author such mysterious disappearances and reappearances of colonies at a locality—as recorded in the case of *Embletonia*—can be justifiably attributed to fatal environmental factors such as lack of food, extremes of temperature or the predominance of other life forms predaceous on nudibranchs. According to the same author, Hecht (1895), dealing with *Aeolidia papillosa*, concluded that death follows very shortly after spawning. However, several individuals of *Aeolida papillosa* kept in the laboratory aquaria were reported by Chambers (1934) to deposit their spawn and in no case did death follow oviposition.

In the light of our results, death did occur when the animals were deprived of any supply of suitable food materials, and did not result after reproduction and spawning. The latter processes have successfully been accomplished so long as food, temperature and other environmental conditions are favourable. In no case, whatsoever, did death follow oviposition in our aquaria, but migration may follow spawning in their natural habitat. Our observations, therefore, corroborate Chambers' view and shows that Hecht's conclusion—cited above—may be speculative rather than experimental.

Summary

1. Nudibranchs exercise considerable choice in the selection of their food. There is a complete chain of conditions from animals feeding exclusively on animal diet to animals depending more or less completely on algae for their food supply.

2. Deprived of all other kinds of food, nudibranchs can depend to a certain extent on suitable planktonic organisms for their diet.

3. Death of the animals does not follow reproduction and spawning, but results when the animals lack a suitable supply of food materials.

4. Large colonies of certain species may disappear as a result of migration after spawning to places where more favourable conditions of food, etc., prevail for the adults.

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EFFECTS OF FERTILIZATION AND DEVELOPMENT ON THE OXIDATION OF CARBON MONOXIDE BY EGGS OF STRONGYLOCENTROTUS AND URECHIS AS DETERMINED BY USE OF C^{13} [1]

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In a previous publication, Black, Epstein and Tyler (1958) presented evidence, obtained by the use of a C^{13} label, for the oxidation of CO in fertilized eggs of the gephyrean worm, *Urechis caupo*. In the light the oxidation of CO is superimposed on the ordinary respiration, so that manometrically an excess gas-uptake is observed. The eggs also oxidize CO in the dark, and this masks, in manometric experiments, an inhibition of respiration by this compound.

Studies by other workers showing excess respiration in the presence of CO, as well as oxidation of this compound, were previously discussed (Black *et al.*, 1958; Rothschild and Tyler, 1958). Pertinent experiments, showing such excess gas-uptake in the presence of CO, have been performed on eggs of the sea urchin (Runnström, 1930; Lindahl, 1939; Rothschild, 1949) and the ascidian (Minganti, 1957), on diapausing grasshopper and silkworm embryos (Bodine and Boell, 1934; Wolsky, 1941; Kurland and Schneiderman, 1959), on skeletal and heart muscle of the frog and rat (Fenn and Cobb, 1932a, 1932b; Schmitt and Scott, 1934; Clark, Stannard and Fenn, 1950), on Earle's L-strain cells from the mouse and MK II cells from monkey kidney cultured *in vitro* (Dales and Fisher, 1959), and on leaf tissue of the wild plum (Daly, 1954).

In the early studies on muscle tissue of the frog and rat noted above, the evidence for the oxidation of CO was obtained by manometric methods alone. Similar evidence has been presented by Dales and Fisher for mammalian cells cultured *in vitro*, and by Allen and Root (1957) for whole blood of rats, dogs and men. The use of isotopically labelled CO has provided direct proof of its oxidation in vertebrate muscle tissues (Clark, Stannard and Fenn, 1950) and in whole turtles and mice (Clark *et al.*, 1949). The use of C^{13}-labelled CO also enabled Black *et al.* (1958) to obtain the first direct evidence for its oxidation in marine eggs.

With regard to previous reports of respiratory stimulation by CO in marine eggs, several points of interest may be mentioned. In the sea urchin Lindahl (1939) found a stimulation of respiration of unfertilized eggs by CO both in the light and in the dark. During development, the excess respiration in the light showed a decrease relative to the total respiration. In the dark, the respiration became progressively more inhibited in the presence of CO as development proceeded. A

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similar finding was reported by Minganti (1957) for the eggs of *Phallusia*. These investigators, as well as Runnström (1956), have rejected the possibility of the oxidation of CO in these materials, while Rothschild (1949) considered this to be the most likely mechanism for the excess respiration in the sea urchin.

It was of interest, then, to determine, by use of the C^{13} label, whether or not sea urchin eggs also possess the capacity to oxidize CO and to follow the changes in this property during development in both the sea urchin and *Urechis*.

If one interprets the data of Lindahl (1939) and Minganti (1957) on the premise that CO-oxidation accounts for the excess gas uptake in CO-O_2 mixtures in the light, then their results indicate that during development this capacity decreases relative to the total respiration. The present results with C^{13}-labelled CO show such relative decrease in capacity for CO-oxidation. These experiments also provide data concerning the effect of illumination on the rates of CO-oxidation at various stages in development, and this pertains to the interpretation of CO-inhibition experiments in general.

Material and Methods

Eggs and embryos of the gephyrean worm *Urechis caupo* and the sea urchin *Strongylocentrotus purpuratus* were employed in the experiments. Non-swimming stages were washed by settling in CO_2-free sea water buffered at pH 8.0 with glycylglycine. Top-swimming embryos were collected by filtration through a coarse sintered-glass filter and washed by low-speed centrifugation.

Gas-uptake and CO_2-production were measured with Warburg-Barcroft manometers. In most experiments the vessels employed were of the type designed by Stanley and Tracewell (1955), with two side-arms in the form of hollow stopcocks, which could be closed off from the main chamber, as well as a conventional vented side-arm. In most experiments the vessels contained 3 ml. of egg suspension, 0.3 ml. of 1 N NaOH ("CO_2-free") in the conventional side-arm, 0.4 ml. of 85% phosphoric acid in one stopcock side-arm which was open during the experiment, and 0.3 ml. of "CO_2-free" alkali in the other stopcock side-arm which was closed during the respiration measurement. At the end of an experiment the acid and alkali from the open side-arms were tipped into the main chamber, in order to obtain a measure of the total CO_2 present. After the measurement had been obtained, the closed side-arm containing alkali was opened, allowing the CO_2 to be taken up in the alkali for subsequent analysis of the C^{13}-to-C^{12} ratio. In some experiments conventional vessels were employed, and acid only was tipped in at the end of the run. The alkali was then transferred quantitatively to the Stanley-Tracewell vessels for measurement and recovery of CO_2. In all the instances, retained CO_2 was measured at the beginning of the experiment by tipping acid and alkali into the main chamber of appropriate vessels at the time of the first manometer reading.

The labelled carbon monoxide was prepared from barium carbonate containing 3.85% C^{13}. This was obtained from the Stable Isotopes Division of the Oak Ridge National Laboratories. The method employed was a modification of that described by Bernstein and Taylor (1947), as outlined in a previous paper (Black *et al.*, 1958). In this method the CO_2 generated from the $BaCO_3$ is passed over a zinc dust-asbestos fiber mixture heated to 520° C. in a combustion furnace. The CO

formed is mixed with unlabelled CO and stored over 0.1 N NaOH in a gas-bulb attached to a leveling bottle. After storage over alkali for several days, samples of the CO were taken from the vessel and re-oxidized by passing over copper oxide at 400° C. The CO_2 produced was collected in a small volume of alkali and the C^{13}-to-C^{12} ratio was determined. In all cases the expected ratio was obtained, indicating practically complete conversion of the original labelled CO_2 to CO. Duplicate samples taken two weeks apart from one batch of CO indicated that there was no loss of the CO upon storage over the dilute alkali.

After attachment of the Warburg vessels to their manometers the experimental vessels (two in most experiments) were connected to a manifold so that they could be gassed with oxygen simultaneously. Each vessel was flushed with one liter of CO_2-free oxygen from a measuring bottle. The two vessels were then evacuated simultaneously by means of a Toepler pump to ⅕ atmosphere, and refilled with the labelled CO, as previously described. These procedures required about 30 minutes, and 10 minutes were allowed for equilibration of the vessels in the water bath. Control vessels were left open to the air or gassed with 80% CO–20% O_2 during this time. The experiments were run at 19° C. Shaker speed was 95 c.p.m. at 3 cm. stroke. Illumination was provided by a bank of 30-watt reflector-type G.E. incandescent lamps located below a glass shelf of the water bath. The intensity of the light at the level of the egg suspensions was 1100–1200 foot-candles. For measurement of respiration and CO-oxidation in the dark, one experimental vessel was placed in a lined black bag.

Determination of the C^{13}–C^{12} ratios of the respired CO_2 were made with a Nier mass-spectrometer (Nier, 1947), modified for the detection of small differences in C^{13}–C^{12} ratio (McKinney *et al.*, 1950).[3] In the experiments with eggs of the sea urchins, the CO_2 which was contained in the alkali of the Stanley-Tracewell vessel was diluted with appropriate amounts of a $NaHCO_3$ solution (usually 0.3 ml. of 0.24 *M*) in order to lower the C^{13}–C^{12} ratio to a range that would be most effectively handled with least danger of "contaminating" the mass-spectrometer.

The alkali and bicarbonate were transferred under CO_2-free atmosphere to reaction vessels wherein the CO_2 was liberated for analysis in the mass-spectrometer. Data obtained in the mass-spectrometer were expressed as deviation of the C^{13}–C^{12} ratio of the sample from that of the standard in parts per mil., *i.e.*,

$$\frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000.$$

The values obtained in this manner were used in isotope dilution equations for the determination of the amounts of CO_2 which had been derived from the C^{13}-labelled CO.

Results

Manometric data

The rates of gas-uptake and of CO-oxidation in the light and in the dark are presented in Table I for the developmental stages of *Urechis* and in Table II for those of *Strongylocentrotus*.

[3] The authors are indebted to Mr. Joop Goris for analyzing some of the samples, and to the Department of Geochemistry for the use of the mass-spectrometer.

TABLE I

Rates of respiration and of CO-oxidation in 80% CO/O_2 in light and dark by eggs of Urechis caupo at early and later stages of development

Expt.	Period of development (hours after fertilization)	Gas-uptake ($mm.^3/10^6$ eggs/hr.)			Excess gas-uptake in 80% CO/O_2 ($mm.^3/10^6$ eggs/hr.)		CO oxidized, from determinations of C^{13}/C^{12} by mass-spectrometry ($mm.^3/10^6$ eggs/hr.)	
		In air (light)	In 80% CO/O_2					
			Light	Dark	Light	Dark	Light	Dark
1	1–8	94	150	125	+56	+31	43.2	21.4
2	20–25	663	839	476	+176	−187	105.6	11.7
3	24–30	724	789	395	+65	−329	89.9	4.6
4	24–30	551	593	327	+42	−224	75.8	5.8
5	52–55.5	853	872	384	+19	−469	65.0	6.6
6	52–55.5	792	815	358	+23	−434	60.6	4.8
7	54–57.5	847	883	384	+36	−463	57.5	4.1

In both species, at all stages studied, the measurements show an increase in rate of gas-uptake of the eggs or embryos in 80% CO/O_2 in the light over those in air. In *Urechis* the amount of excess rate of gas-uptake declines somewhat during development, while in *Strongylocentrotus* it increases slightly. However, in both species,

TABLE II

Rates of respiration and of CO-oxidation in light and dark by eggs of Strongylocentrotus purpuratus, unfertilized and at early and later stages of development

Expt.	Period of development (hours after fertilization)	Gas-uptake ($mm.^3/10^6$ eggs/hr.)			Excess gas-uptake in 80% CO/O_2 ($mm.^3/10^6$ eggs/hr.)		CO oxidized, from determinations of C^{13}/C^{12} by mass-spectrometry ($mm.^3/10^6$ eggs/hr.)	
		In air (light)	In 80% CO/O_2					
			Light	Dark	Light	Dark	Light	Dark
1	0 (unfertilized)	16.0	29.9	28.5	+13.5	+12.3	8.9	8.0
2	0 (unfertilized)	22.5	40.9	37.5	+18.4	+15.0	13.4	10.6
3	1–5	69.9	97.4	88.3	+27.5	+18.4	20.5	9.9
4	1–7	81.9	118.4	105.0	+36.5	+23.1	24.1	10.8
5	26–30	166.0	190.0	132.0	+24.0	−34.0	25.0	3.4
6	27.5–31.5	200	238	203	+38.0	+3.0	25.0	9.8
7	46–50	211	242	164	+31.0	−47.0	24.4	3.3
8	49–53	264	321	216	+57.0	−46.0	34.0	6.4

the relative excess gas-uptake with respect to the respiration in air is considerably lower in the later embryonic stages than in the unfertilized or freshly fertilized eggs. Thus for *Urechis* the average values of the percentage excess gas-uptake, in 80% CO/O_2 in the light, relative to the respiration in air, are as follows:

	Stage		
	Freshly fert. (1–8 hrs.)	1 day (20–30 hrs.)	2 day (52–57.5 hrs.)
% excess gas-uptake (light)	60	14	3

For *Strongylocentrotus* the average values are:

	Stage			
	Unfertilized	Freshly fert. (1–7 hrs.)	1 day (26–31.5 hrs.)	2-day (46–53 hrs.)
% excess gas-uptake (light)	83	43	17	18

In the dark the eggs of both species show greater gas-uptake in the CO-O_2 atmosphere than in air for the early stages of development and considerable inhibition at the later stages. For *Urechis* the average values of the percentage excess gas-uptake in 80% CO/O_2 in the dark relative to the respiration in air are:

	Stage		
	Freshly fert.	1 day	2 day
% excess gas-uptake (dark)	+33	−39	−55

For *Strongylocentrotus* the average values are:

	Stage			
	Unfert.	Freshly fert.	1 day	2 day
% excess gas-uptake (dark)	+71	+28	−10	−20

These results accord with those of Lindahl (1939) on the sea urchin showing, in CO in the dark, excess gas-uptake for the unfertilized and freshly fertilized eggs,

TABLE III

Manometrically measured respiratory quotients of eggs and embryos of Urechis and Strongylocentrotus in air and in CO

Experiment	R.Q. in air	Apparent R.Q. in 80% CO/O_2 in the light	Apparent R.Q. in 80% CO/O_2 in the dark
Strongylocentrotus			
1	0.58	0.63	0.73
2	0.85	0.65	0.77
3	0.81	0.77	0.83
4	0.77	0.71	0.85
5	0.85	0.85	0.92
6	0.76	0.77	0.81
7	0.84	0.91	1.0
8	0.81	0.81	1.04
Urechis			
1	0.96	0.98	0.99
2	0.86	1.05	1.0
3	0.82	1.0	1.0
4	0.81	0.92	0.99
5	1.02	0.96	1.08
6	0.81	0.82	1.00
7	0.91	0.92	1.10

and inhibition of respiration for the later embryos. Also, the results obtained in CO in the light accord with those of Lindahl (1939) on sea urchin eggs and Minganti (1957) on ascidian eggs, which showed that the relative excess gas-uptake decreased progressively as development proceeded.

Determinations of CO_2-production were made, as indicated under *Methods*, in these same experiments. Division of these values by the corresponding total gas-uptake gives the respiratory quotients (R.Q.) and apparent respiratory quotients (A.R.Q.) listed in Table III. The A.R.Q. refers to the experiments done in presence of CO since the occurrence of CO-oxidation means that some of the gas-uptake represents CO. Such oxidation has an R.Q. of 2, but manometrically it would be recorded as an R.Q. of 0.67. One would therefore expect that the values for the A.R.Q. in the vessels run in presence of CO would be lower than the R.Q. values in air wherever CO-oxidation occurred.

TABLE IV

Ratio of dark-to-light CO-oxidation

	Rate of CO-oxidation in the dark divided by the rate in the light	
	In *Urechis*	In *Strongylocentrotus*
Unfertilized egg	—	0.79
		0.79
Fertilized egg	0.50	0.48
		0.45
One-day larvae	0.11	0.39
	0.05	0.13
	0.08	
Two-day larvae	0.10	0.18
	0.08	0.13
	0.07	

The data, however, are mostly in the opposite direction. This would indicate that along with an inhibition of ordinary respiration (in the dark) and its own oxidation, the CO may be inducing a relative stimulation of glycolysis, such as has been reported by Daly (1954) for wild plum, Ducet and Rosenberg (1952) for spinach, Marsh and Goddard (1939) for carrot, Laser (1937) for rat retina and mouse chorion, and Dales and Fisher (1959) for mouse L-strain cells. The data also bear on the question of possible CO_2-assimilation. This is considered in the *Discussion*.

Data from mass spectrometer

The data for the rates of oxidation of CO in the light and dark by the developmental stages investigated are presented in columns 8 and 9 of Tables I and II.

For *Urechis*, the rate in the light is about twice as high in 20–30 hour larvae as in the cleaving eggs. In 50–58 hour larvae, the rate is about one and one-half times that of the fertilized egg.

In the dark, the rate of CO-oxidation in *Urechis* (Table I, column 9) is nearly 4 times greater in cleaving eggs than in the swimming embryos.

For *Strongylocentrotus* the rate of CO-oxidation in the light in fertilized eggs is about twice that of the unfertilized eggs. Following the striking change after fertilization, there is not much increase in CO-oxidation in the light during the remainder of the developmental period investigated. In the dark (column 9) there is no major change in CO-oxidation after fertilization and during later development.

A comparison of CO-oxidation in the dark with that in the light is given in Table IV in terms of the ratio of the two rates at the various developmental stages. In both species this ratio decreases in the later stages. Interpretation of this is offered below on the basis of change in the rate of electron transfer by cytochrome oxidase during development.

For comparison of the rates of CO-oxidation with the excess respiration it should be noted that for each mole of CO_2 derived from CO there would be a manometrically measured gas-uptake of one and one-half moles, according to the equation $CO + \frac{1}{2} O_2 \rightarrow CO_2$. If it is assumed that there is no inhibition of the ordinary respiration by CO in the light, then one would expect the values obtained by mass-spectrometry (column 8 of Tables I and II) for CO-oxidation to equal two thirds of the excess gas-uptake determined manometrically (column 6 of Tables I and II). Such an agreement was found in the previously reported (Black *et al.*, 1958) experiments on freshly fertilized eggs of *Urechis*. It is also found in the present data for *Strongylocentrotus* at all stages and the data for *Urechis* at the early stages. However, for *Urechis* at the 52–57.5 hour stage the excess gas-uptake is considerably lower than expected from the values for CO-oxidation. This evidently means that under the conditions of the experiment, at this stage, there is an inhibition of the ordinary respiration by CO in the light. Possibly this is correlated with the high absolute rate of respiration that the *Urechis* embryos exhibit at this stage. Whether or not increase in illumination would overcome the deficiency in excess gas-uptake (in comparison with CO-oxidation) in *Urechis* at this stage has not as yet been determined.

Discussion

Respiratory inhibition by CO

The mass-spectrometric data for CO-oxidation in the dark permit evaluation of "true" CO-inhibition of ordinary respiration. These values for the inhibition are presented in Table V and are derived simply by subtracting one and one half times the values in column 9 of Tables I and II from the corresponding values in column 5. This is then the respiration in the presence of CO in the dark corrected for CO-oxidation, and its percentage difference from the respiration in air (column 3) gives the inhibition values listed in Table V.

These data show no inhibition of ordinary respiration by CO in the dark for unfertilized and freshly fertilized eggs of *Strongylocentrotus* and for freshly fertilized eggs of *Urechis*. For the later stages of both species there is inhibition of ordinary respiration, the amount of which is, in general, greater the greater the absolute rate of respiration of the air-controls.

A comparison of the data showing lack of inhibition with those of related experiments reported in the literature would be difficult in view of uncertainty, in earlier work, as to the extent to which CO-oxidation occurs in the dark. In any case the lack of inhibitory action of CO in the dark on the early developmental

stages does not necessarily imply the absence of a cytochrome system. As Warburg (1927) pointed out, one would not expect a cell to show much inhibition of respiration in the presence of CO unless it was rapidly oxidizing substrate. Thus Runnström (1930, 1932) and Örström (1932) found that when the respiratory rate of *Paracentrotus* eggs was increased by means of dimethylparaphenylene diamine it could be inhibited by CO. Other evidence for the operation of a cytochrome system in eggs of *Urechis* has been previously presented (Rothschild and Tyler, 1958). A brief review is given there, and a more extended one by Runnström (1956) deals with this system in sea urchins.

TABLE V

"True" respiratory inhibition by CO in the dark in developmental stages of Urechis and Strongylocentrotus, obtained after correcting for CO-oxidation

Period of development	Percentage inhibition of respiration by CO in the dark	
	In *Urechis*	In *Strongylocentrotus*
Unfertilized egg	—	0
		0
Fertilized egg	0	−5.0
		−9.0
One-day larvae	34	23.5
	46	6.0
	42	
Two-day larvae	56	24.6
	56	21.8
	55	

Rothschild (1949) reported an inhibitory effect (Av. 38%) of light on the respiration of unfertilized *Psammechinus* eggs and a smaller effect (Av. 9%) on that of the fertilized eggs. This effect was not found with fertilized eggs of *Urechis* (Rothschild and Tyler, 1958), nor with unfertilized or fertilized eggs of *Phallusia* (Minganti, 1957). It has not, as yet, been investigated with eggs of *Strongylocentrotus*. If such effect occurs with this material, the correction for it would be in the direction that would show an inhibitory action of CO on the respiration of the unfertilized or early stage eggs of this species.

Oxidation of CO

The values given for rates of oxidation of CO in Tables I and II involve the assumption that no CO-fixation occurred and that there was no assimilation of the CO_2 derived from CO in any of the developmental stages. Hultin and Wessel (1952) have found that CO_2-fixation occurs in all stages of the development of the sea urchin pluteus. The extent to which metabolic CO_2 was assimilated in our experiments is not known. If assimilation occurs, then the correction for this would result in larger values for CO-oxidation than those presented here.

From the R.Q. data presented it appears that assimilation cannot account for more than a small fraction of the metabolically produced CO_2. If we assume unity as the expected value of the R.Q. in the absence of assimilation and take 0.85 as a representative value obtained in the present experiments, then there could be

15% CO_2 assimilation. The values for CO-oxidation could then be that much greater than listed.

The participation of Warburg's "iron-containing respiratory ferment" in the oxidation of CO was postulated by Fenn and Cobb (1932b). Subsequently, Breckenridge (1953), using C^{14}-labelled CO and both crude and purified preparations of cytochrome oxidase from heart muscle of the pig, showed that this enzyme is indeed responsible for the oxidation of CO, in the presence of cytochrome *c* and hydroquinone. Evidence somewhat similar to that of Breckenridge is presented in an accompanying paper (Black and Tyler, 1959) for the participation of cytochrome oxidase in CO-oxidation by eggs of the sea urchin.

Breckenridge has based his explanation for the oxidation of CO on the hypothesis that three molecules of cytochrome *a* and one of a_3 are combined in a single unit, as proposed by Ball *et al.* (1951). According to Breckenridge, if as many as three iron atoms of a tetraheme unit are oxidized and the fourth is reduced and combined with CO, then the CO can be oxidized. On the other hand, if as many as three of the iron atoms are reduced, combination of a_3 with CO will inhibit cytochrome oxidase activity (and presumably the oxidation of CO). He has cited evidence to show that such an inhibition would be expected when the rate of electron-transfer is high (*i.e.*, when cytochrome *c* and reducing substrate are present in excess).

Breckenridge has indicated that under conditions of rapid electron transfer, one might expect a relatively large fraction of the iron to be reduced as compared with the fraction when electron transfer is slow. In the dark, nearly all the reduced iron of a_3 would combine with CO, whereas in the light, even when electron transfer is rapid, only a small fraction of the a_3-iron would be associated with CO, the proportion depending on the intensity of light. Under these conditions, therefore, in the light, CO-oxidation would be accelerated, up to a point, by increasing rates of electron transfer, whereas in the dark, the oxidation of CO would be inhibited when the electron transfer is rapid. The ratio of dark-to-light oxidation would thus decrease with increasing rates of electron transfer. Such a decrease in ratio is found in the developing eggs of *Urechis* and *Strongylocentrotus* as the respiratory rate increases (Table III). If one accepts the above interpretation, it may tentatively be concluded that the rate of electron transfer by the cytochrome system of unfertilized eggs and of early developmental stages is low; *i.e.*, the cytochrome a-a_3 unit is highly "unsaturated" with reducing substrate. This interpretation is consistent with the lack of respiratory inhibition by CO in these stages in the dark. During later development, the cytochrome oxidase molecules become more nearly "saturated" with reducing substrate, leading to more rapid electron transfer, greater respiratory inhibition by CO in the dark, and a lower rate of oxidation of this compound. This interpretation has been used by other authors to explain the differential effects of CO on the respiration of diapausing and non-diapausing silkworm embryos (Kurland and Schneiderman, 1959) and of developing eggs of the sea urchin (Runnström, 1930).

Summary

1. The rates of gas-uptake and oxidation of CO (labelled with C^{18}) have been determined for developing eggs of the gephyrean, *Urechis caupo,* and for unferti-

lized eggs and developing embryos of the sea urchin, *Strongylocentrotus purpuratus*. In the light in 80% CO/20% O_2, there is excess gas-uptake over that of the air-controls at all stages of development, but the percentage of excess uptake falls off as development proceeds. In the dark there is increasing inhibition of respiration by CO during development.

2. The rate of CO-oxidation in the light increases less than two-fold in *Urechis* during 50 hours of development. In *Strongylocentrotus* the rate of CO-oxidation in the light nearly doubles after fertilization, but increases relatively little during later development.

3. In the dark, in *Urechis*, the rate of CO-oxidation during early cleavage is about half that in the light, but the dark-rate decreases five-fold during the swimming stages. In the sea urchin there is only a slight decrease in rate of CO-oxidation during development. The developmental changes in the ratio of dark-to-light oxidation of CO are interpreted on the basis of an increasing degree of saturation of cytochrome oxidase with its reducing substrate.

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CYTOCHROME OXIDASE AND OXIDATION OF CO IN EGGS OF THE SEA URCHIN STRONGYLOCENTROTUS PURPURATUS[1]

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Black and Tyler (1959) have shown that developing eggs of the sea urchin and the gephyrean worm *Urechis* can oxidize CO, and these authors have presented data on the inhibitory effects of CO on respiration of developing embryos. The rates of CO-oxidation and ordinary respiration were such that in the presence of CO an excess gas-uptake occurred in the light in early developmental stages, but the percentage of excess uptake diminished as the respiratory rate increased. In the dark there was increasing inhibition of respiration and, in *Urechis*, diminishing CO-oxidation as the respiratory rate increased.

The inhibitory effect of CO on cytochrome oxidase is well-known. Fenn and Cobb (1932) were the first to suggest that cytochrome oxidase could also oxidize this inhibitor, and Breckenridge (1953) obtained direct proof of this phenomenon, using labelled CO and purified cytochrome oxidase from pig heart. In the latter study, the author suggested a relationship between the relative proportions of oxidized and reduced iron atoms of cytochrome oxidase and the efficiency of CO-oxidation.

In order to interpret the data obtained on CO-oxidation in embryos, it was necessary to determine whether cytochrome oxidase was in the pathway of CO-oxidation in this material, and if so, whether the rates of CO-oxidation in light and dark by enzyme preparations could be changed by altering the amounts of cytochrome *c* in the system. It will be shown that cytochrome oxidase is involved in CO-oxidation in sea urchin eggs, and that the relative rates of oxygen-uptake and CO-oxidation can be altered by changing the concentration of cytochrome *c*.

Materials and Methods

Granular preparations of active cytochrome oxidase were made by centrifugation of homogenates of unfertilized, jelly-free eggs of *Strongylocentrotus purpuratus* in 0.1 *M* phosphate, made to pH 7.4 with "CO_2-free" NaOH. The eggs were repeatedly forced through a No. 18 needle into 30 volumes of buffer, and the homogenate was transferred quantitatively to centrifuge tubes. The preparation was centrifuged at approximately 44000 × gravity in an angle head of the Spinco ultracentrifuge for 20 minutes. The sedimented granules were re-suspended in fresh buffer and centrifuged again at the same acceleration. The final sediment was suspended in 0.1 *M* buffer, pH 7.4, to give a concentration of 10%, based on

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original egg volume. Quantitative transfers of homogenates, combined with counts made on aliquots of the original egg suspension, made it possible to base the values obtained for cytochrome oxidase activity and CO-oxidation on egg-numbers, since it could be assumed that a constant percentage of the total activity was recovered in separate runs.

The enzyme activities were measured for one hour in Warburg vessels of the type devised by Stanley and Tracewell (1955), and the CO_2 was measured and recovered for C^{13}/C^{12} analysis. The procedures involved in the measurement of gas-uptake and CO-oxidation have been described (Black and Tyler, 1959). The vessels were illuminated in these experiments by a bank of 150-watt G.E. floodlamps, providing an intensity of 3500 to 5000 foot candles at the level of the enzyme suspensions. In the dark experiment, the measurements were made at night.

TABLE I

Rates of CO-oxidation by granule-preparations in the presence and absence of cytochrome c and ascorbate. Final concentrations, where appropriate, were as follows: egg granul s, 6% (based on egg volumes); cytochrome c, 2×10^{-4} M; ascorbate, 0.02 M. All vessels contained PO_4, 0.06 M, pH 7.4, and $AlCl_3$, 8×10^{-4} M. Experiments were run in light.

Experiment	Contents of vessel	CO oxidized (mm.³/10^6 eggs/hr.)
1	Ascorbate + cytochrome *c* (blank)	1.3
	Granules only	2.4
	Granules + ascorbate	5.5
	Granules + ascorbate + cytochrome *c*	11.3
2	Granules + cytochrome *c*	3.2
	Granules + ascorbate	4.8
	Granules + ascorbate + cytochrome *c*	20.0

RESULTS

The rates of CO-oxidation by two granule-preparations in the presence and absence of excess ascorbic acid and excess cytochrome *c* are presented in Table I. It can be seen that rates of CO-oxidation are very low in preparations lacking either ascorbate or cytochrome *c*. A low rate of CO-oxidation was also found in the blanks containing ascorbate and cytochrome *c*.

In the presence of both cytochrome *c* and ascorbic acid, rates of CO-oxidation comparable to those of unfertilized eggs and early developmental stages are found. It may be concluded that both these substances are necessary for the efficient oxidation of CO by the granule-preparations.

In Table II, the rates of gas-uptake and CO-oxidation in light and dark are listed for enzyme preparations at three different concentrations of cytochrome *c*. It can be seen that at cytochrome concentrations giving an O_2-uptake of ½ to ¼ the maximum rate, the rate of CO-oxidation in the light is actually higher than that in the vessels containing excess cytochrome *c*. Furthermore, in those vessels in which cytochrome *c* is limiting the rate of O_2-uptake, there is an excess gas-uptake in CO in the light. In the presence of excess cytochrome *c* the O_2-uptake is inhibited by CO in the light. It is of interest that Breckenridge (1953) also obtained excess gas-uptake in CO by his cytochrome oxidase preparations. In the dark the O_2-uptake is strongly inhibited by CO at all three concentrations of

cytochrome *c*, but the percentage inhibition is greatest at high concentrations of cytochrome *c*. There is not much difference between the rates of CO-oxidation in the dark at different cytochrome concentrations.

Discussion

It can be concluded from the data of Tables I and II that added cytochrome *c* is necessary for the oxidation of CO by cytochrome oxidase, but the concentration of cytochrome *c* which gives maximum O_2-uptake is higher than the optimum concentration for CO-oxidation in the light.

Table II

Rates of gas-uptake and CO-oxidation by granule preparations in the light and dark. All rates are corrected for the autoxidation in the ascorbate-cytochrome c blanks. The vessels contained PO_4, 0.06 M, pH 7.4; $AlCl_3$, 8×10^{-4} M; ascorbate, 0.02 M, and homogenate, 6% (based on egg volumes). Final concentrations of cytochrome c are given below.

Experiment	Cytochrome *c* concentrate in moles/liter	Gas-uptake (mm.³/10⁶ eggs/hr.)				CO oxidized (mm.³/10⁶ eggs/hr.)	
		Light		Dark		Light	Dark
		Air	80% CO/O_2	Air	80% CO/O_2		
1	2×10^{-4}	762	462			16.3	
	4×10^{-5}	364	382			26.0	
	2×10^{-5}	187	254			21.4	
2	2×10^{-4}	779	495	639	61	16.8	6.1
	4×10^{-5}	356	408	357	139	26.5	6.1
	2×10^{-5}	192	244	217	125	24.0	6.3

The data are of some value in interpreting the changes in CO-oxidation by developing eggs. Black and Tyler (1959) have postulated that in the light, an increasing rate of electron-transfer by the cytochrome system would enhance CO-oxidation up to a point. A comparison of rates of CO-oxidation in the light and no added cytochrome (Table I) with that when there is enough added cytochrome to give ½ maximum O_2-uptake (Table II) shows that the rate of CO-oxidation is increased about 5-fold by this increase in electron transfer. However, at very high rates of activity (excess cytochrome *c*) the rate of CO-oxidation falls. This is in accord with the suggestion of Breckenridge (1953) that CO would inhibit cytochrome oxidase (and presumably CO-oxidation) when a relatively high proportion of the iron of cytochromes *a* and a_3 became reduced, as it might in the presence of excess cytochrome *c* and excess reducing substrate. It can be seen from Table II, column 4 that in the presence of excess cytochrome *c* there is inhibition of O_2-uptake by CO in the light.

The similarity between the excess gas-uptake in CO in the light exhibited by the enzyme preparations at low concentration of cytochrome *c* (Table II), and the gas-uptake in CO by whole eggs during early development is worth noting. For the whole eggs, Black and Tyler regarded the results as an indication that

during early development the cytochrome oxidase was "unsaturated" with reducing substrate, as proposed originally by Runnström (1930) on the basis of other experiments. The present finding is in accord with that interpretation.

In the dark, the lack of variation in rate of CO-oxidation at different levels of cytochrome *c* is perhaps not too surprising, when the data are compared with those for intact embryos of the sea urchin, in which the rate of CO-oxidation changes little during development. It should be noted that even at the lowest levels of cytochrome used in the dark, the rate of O_2-uptake by the granules was still much higher than that of early, developing eggs. Presumably if the cytochrome *c* had been reduced still further in these preparations, an excess gas-uptake in CO might have been evident in the dark, as it was in unfertilized and fertilized eggs.

Summary

1. The cytochrome oxidase activity and ability to oxidize CO (labelled with C^{13}) have been determined for sedimented, washed particles obtained from unfertilized eggs of the sea urchin, *Strongylocentrotus purpuratus*. Both added cytochrome *c* and ascorbate were required for the rapid oxidation of CO by the particles, and it is concluded that cytochrome oxidase is in the pathway of oxidation.

2. In the light, the rate of CO-oxidation was found to be greatest at the concentration of cytochrome *c* which gave about ½ the maximum O_2-uptake. When the uptake of oxygen was maximal, the CO inhibited both the O_2-uptake and the CO-oxidation in the light. In the dark the rates of CO-oxidation by the particles were not greatly affected by changes in the level of cytochrome *c*. The results are used as a basis for the interpretation of developmental changes in CO-oxidation in embryos.

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OSMOTIC STUDIES OF AMPHIBIAN EGGS. I. PRELIMINARY SURVEY OF VOLUME CHANGES

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The osmotic properties of eggs of a great many different species have been studied using a wide variety of techniques. The data obtained from such investigations show that on the one hand there are some eggs, such as those of marine invertebrates, which are freely permeable to water and which appear to behave as osmometers when placed in solutions of different osmotic pressures. If the osmotically inactive material within these eggs is taken into consideration, the Van't Hoff-Mariot Law holds over a wide range of osmotic pressures in the form, $P(V - b) = \text{constant}$ in which b is the osmotically inactive material and P and V are the osmotic pressure (or concentration) of the surrounding fluid expressed in appropriate osmotic units, and the volume of the cell, respectively. The review by Lucké and McCutcheon (1932) discusses much of the older literature on the osmotic changes in marine eggs. At the other extreme are eggs of many freshwater fishes, for example, which appear to be surrounded by a membrane that is completely impermeable to water. Gray (1932) reported that trout eggs are impermeable to both water and intracellular electrolytes, and suggested that it was the vitelline membrane which served as the impermeable barrier. Manery and Irving (1935) showed that when unfertilized or fertilized trout eggs were placed in water their weight increased during the first hour, indicating an uptake of water. But after a short exposure to water there is a change in the eggs and a cytoplasmic membrane becomes impermeable to water. Krogh and Ussing (1937) demonstrated that although D_2O could enter trout eggs when they were first placed in a mixture of D_2O and H_2O, after 6 hours the permeability to D_2O was nil. Fertilized eggs remained impermeable to water during the first 13 days of development.

Eggs of various other species of animals are also laid in fresh water and so are faced with the same osmotic problem as are fish eggs. The eggs of amphibians are in this category. Since amphibian embryology has been studied extensively, particularly the development of frog eggs, it is not surprising to find in the older literature a number of attempts to learn something about the way in which a frog egg avoids destruction resulting from the entrance of large quantities of water at the time it is laid. Unfortunately, there are a number of conflicts in these old data so that even today it is not possible to describe accurately and in detail what happens to the eggs of frogs when they are laid in water. That some sort of change does occur is suggested by the fact that frog Ringer (or 0.66–0.70% NaCl), which is usually considered a suitable medium for ovarian eggs, appears to be hypertonic for fertilized eggs. Standard solution (or 0.38% NaCl) is frequently used as a

[1] The authors are indebted to the Rockefeller Foundation for a grant-in-aid.

suspension medium for developing frog eggs. Measurements of freezing point depression and of vapor pressure suggest that a fertilized egg actually contains less osmotically active material than an ovarian egg although there is considerable divergence of opinion of the amount of the change and the mechanism responsible for it. Another point of general agreement seems to be that the fertilized frog egg, unlike the egg of the trout, is not surrounded by a membrane impermeable to water. The increase in volume and/or weight which accompanies development is generally interpreted as resulting from a gradual uptake of water.

This early work is reviewed in some detail by Needham (1931) and to a lesser extent by Holtfreter (1943). Very briefly, Backman (1912) and Backman and coworkers (1909a, 1909b, 1912) measured freezing point depressions and concluded that there was a marked drop in the osmotic pressure of the fertilized eggs of *Bufo vulgaris*. This reached a minimum at the early blastopore stage and then rose again, being slightly less than half the value of the ovarian egg before the blastopore closed. They believed that salts did not diffuse from the eggs but rather that the salts became provisionally adsorbed and so were osmotically inactive. On the basis of diameter measurements, Backman showed that unfertilized eggs did not change volume after several hours of exposure to a solution considerably more hypertonic than a solution in which morulae did not change volume. Subsequent workers, including Bialaszewicz (1912) Przylecki (1917) and Krogh *et al.* (1938), could not confirm the tremendous drop in osmotic pressure at the time of fertilization but did find a smaller drop at about this time. The latter group used a vapor pressure technique for measuring total osmotically active materials and in addition made some measurements of total alkali and of chloride. They believed that changes in "total concentration" could be explained on the basis of the entrance of water during early development. They also believed that some salt might be lost from the egg.

Adolph (1927) was one of the few workers who could find no relation between osmotic pressure of the surrounding solution and the volume of frog embryos. He concluded that during the early stages of development the permeability to water and to solutes was somewhat variable, and that the volume of the egg could not be regulated accurately. Holtfreter (1943) studied the effect of distilled water, tap water, standard solution and frog Ringer on the "outline areas" of the eggs of *R. pipiens*. He first studied unfertilized eggs taken fresh from the oviduct and left in their gelatinous capsules. He found that the volume of these eggs decreased markedly when they were placed in frog Ringer "which ought to be isotonic . . ." (pp. 306–307). When these eggs were placed in water they showed a swelling in 24 hours no greater than the swelling in standard solution. Fertilized eggs in distilled water developed normally. He found no significant difference in osmotic behavior between an unfertilized and a fertilized egg "except that the swelling effect of standard solution seems to be more pronounced in the former. It is suggestive to relate the uniformity of the behavior of the fertilized and the unfertilized eggs to the fact, mentioned before, that in any of these media also unfertilized eggs form a vitelline membrane and a solidified surface layer. The latter is, as shall be shown, a most important factor for regulating the internal osmotic conditions" (p. 307). He continues by stating: "The coat in unferti-

lized eggs is apparently no more permeable than in fertilized eggs. Volumetric changes are therefore a rather unreliable indicator for the actually existing internal osmotic concentration" (p. 308). Fertilized eggs when placed in distilled water shortly after fertilization swell rapidly at first, but following neurulation this rate of swelling decreases. In standard solution the eggs never attain the same initial size as in plain water. In Ringer, these eggs shrink. His data suggest that up to the neurula stage, the osmotic pressure of the eggs is approximately equal to that of standard solution. "The membranes therefore act as an imperfectly regulatory mechanism in establishing an inner milieu which plays the role of an osmotic buffer against the varying outside osmotic pressure" (p. 311). Holtfreter concluded that by the time the eggs were in the oviduct they were isotonic with his standard solution.

The most recent studies of this general nature are summarized by Allende and Orías (1955). This paper reports a rather wide variety of observations concerning the development of frogs that have been made in Houssay's laboratory. The point most pertinent to the present discussion is their suggestion that the change in the resistance to water of the eggs occurs when they are released from the ovary.

The present investigation was undertaken with the hope that it might demonstrate some of the changes which occur in the frog egg before and after it is released from the ovary. The first portion of the work consists of an initial survey of volume changes which occur when ovarian or fertilized eggs are placed in salt solutions of different osmotic pressures. This will then be followed by a more detailed study of the water, sodium and potassium content of eggs. It is thought that perhaps a better understanding of the quantities of these substances in the ovarian, unfertilized and fertilized eggs, and their movements under different conditions may add to an understanding of how these eggs and early embryos "solve their osmotic problems."

Materials and Methods

Since *R. pipiens* does not naturally occur in the vicinity of Bogotá, Colombia, the present studies were made using *Hyla labialis*, the most common local frog, and *Bufo marinus*, a toad which could be readily brought in from the warmer parts of Colombia.

Adult females were selected, the body cavity was exposed by dissection and eggs were removed from the ovary for study. Injections of suspensions of *Bufo* pituitaries in 0.66% NaCl were used to stimulate egg laying and sperm release. Volumes were calculated from diameter measurements made with an ocular micrometer. Since the eggs were essentially spherical, only one diameter was measured and the volume of a sphere with this diameter was calculated. In one experiment the eggs in one solution were not spherical. In this single instance, the largest and smallest diameters were measured and the cells were considered as prolate ellipsoids for volume calculations. In some experiments the eggs with their follicular layers were removed from the ovary and placed directly in approximately 20 cc. of the solution to be studied, in a covered dish. In a few of the experiments, the follicular layers were removed before placing the eggs in the solutions.

Results

Bufo—ovarian eggs

Eggs were removed from the ovary of *Bufo* and no attempt was made to remove the ovarian layers. Twenty-five eggs were placed in each of 5–7 solutions of sodium chloride of concentrations ranging from 0.55–0.75%. Measurements of cell diameters were begun ½–1 hour after the eggs had been placed in these solutions, 1½–2 hours being necessary to complete the measurements. The measurements were then repeated 5–8 hours later (18 hours in one experiment). Only the largest eggs were selected and they yielded sufficiently uniform data so that the

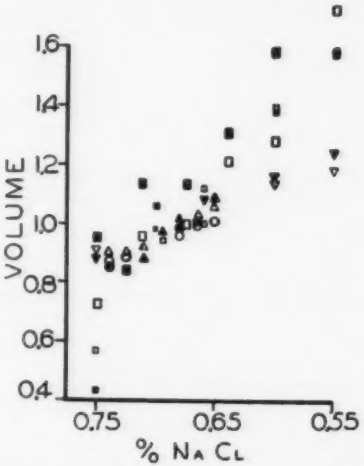

Figure 1. Volume changes of ovarian eggs of *Bufo* (with follicular layers) when placed in solutions of NaCl of different concentrations. Open symbols: measurements made ½–2 hours after eggs were placed in the solutions. Closed symbols: measurements made 5–18 hours after eggs were placed in the solutions.

diameters of the 25 eggs in one solution were averaged and the volume was calculated from this average value. In order to eliminate the variability in the measurements made on eggs from different females, it was assumed that 0.66% NaCl was isotonic with the eggs and the volume in this solution was given the value of 1.00 The volumes in the other solutions were then divided by the volume in 0.66% NaCl to obtain relative volumes. Typical data are presented in Figure 1. It can be seen that in solutions more hypertonic than 0.66%, there is a shrinking of the eggs, and in more hypotonic solutions, a swelling. In the majority of the experiments, the volumes after several hours' exposure to the solutions were approximately the same as those after the eggs had been in the solutions for approximately an hour.

These data were analyzed in the following manner to see if the eggs were behaving as osmometers. The formula $P_1(V_1 - b) = P_2(V_2 - b)$ was used. The

relative values were substituted for V_1, V_2, etc. (*i.e.*, each volume was divided by the volume in 0.66% NaCl) and the relative osmotic pressures for P_1, P_2, etc. (*i.e.*, 0.66% was considered to have one unit of osmotic pressure and the other percentages were divided by 0.66 to give relative values of osmotic pressure). In general, these calculations showed that the larger volume changes, such as were measured in the curves indicated by open and closed rectangles in Figure 1, were greater than could be explained on the assumption that the eggs were behaving as simple osmometers (*i.e.*, negative values of b were obtained). The smaller volume changes observed (such as those between 0.75 and 0.65%) would be predicted if the cells were osmometers with a relatively small b-value. Since there is considerable error in the selection of 100 or more eggs all of the same size, which is necessary for such an experiment, and since there is considerable error in the

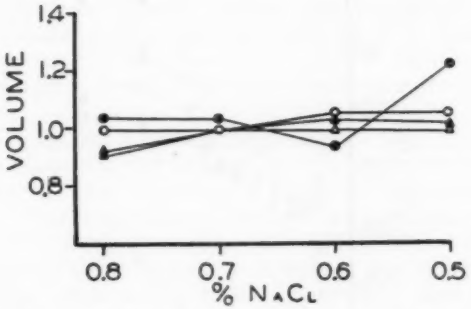

FIGURE 2. Volume changes of ovarian eggs of *Bufo* separated from the follicular layers, when placed in solutions of NaCl of different concentrations. Open symbols: measurements made immediately after eggs were placed in the solutions. Closed symbols: measurements made one hour after eggs were placed in the solutions.

diameter measurements, it is difficult to quantitate such data. However, it seems reasonable to conclude that these eggs (with their surrounding follicular layers) have a tendency to swell as much or more than would be predicted if they were behaving as simple osmometers. There is no evidence that a large fraction of their volumes (b) is reacting as inert to osmotic pressure changes.

A few measurements were then made using ovarian eggs separated from the follicular layers using jeweler's forceps. Because of the time involved in preparing such eggs, only 6 or 8 eggs were measured and averaged in each solution. The outer layers were removed in 0.66% NaCl, the egg was then transferred to the appropriate solution, the final layer was removed and the diameter was measured immediately. These measurements were repeated at the end of an hour. Measurements made after the eggs had been in the solutions for several hours were not satisfactory due to the distortion of the eggs. The data obtained from two experiments are presented in Figure 2. It can readily be seen that except for one point, the volume changes are considerably smaller than in the preceding experiment (the same scale is used in all of the figures). When these data are treated in the same way as those previously, except for the one large difference

between 0.50 and 0.60% NaCl in the one curve, all of the values of *b* are positive and large (60–80%). This is to say, these cells are behaving as simple osmometers with a large portion of their volume being osmotically inactive. Another observation which adds support to this conclusion was the following. When one of these eggs which had become shrunken and distorted in 0.8% NaCl was placed in 0.5% NaCl, it increased in volume and once again became spherical. More experiments are needed to analyze in more detail the difference between the volume changes of ovarian eggs with and without their layers. However, the present data might be interpreted as indicating that the ovarian egg of *Bufo* changed volume very little in solutions of different osmotic pressures, but the follicular layers surrounding it change volume to a much greater extent in these solutions.

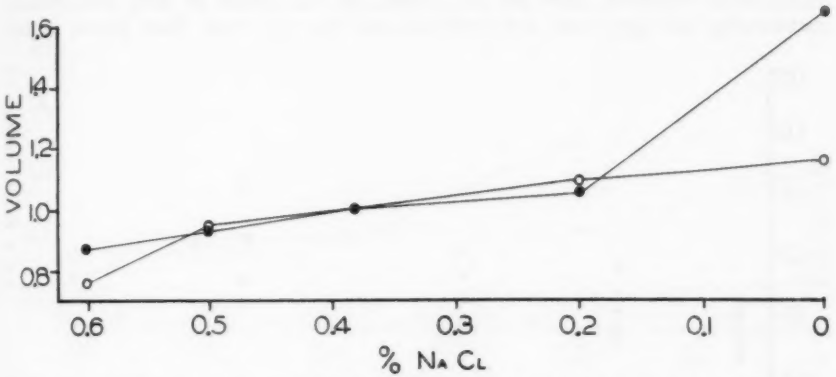

FIGURE 3. Volume changes of fertilized eggs of *Bufo* (with jelly layer) when placed in solutions of NaCl of different concentrations. ○, measurements made after 1½–4 hours; ●, after 24 hours.

Bufo—fertilized eggs

Two experiments were performed using fertilized eggs of *Bufo*. The eggs were laid in tap water and subsequently were separated from the jelly and placed in the appropriate solutions. The layer of jelly immediately surrounding the eggs was not removed. In one experiment the measurements were made between 1½ and 4 hours after placing the eggs in the solutions. The same values were obtained when the measurements were repeated after 8 hours of exposure to the solutions. In the second experiment the measurements were made after the eggs had been in the solutions for 24 hours. In these two experiments the eggs were in blastula or earlier stages. The data are presented in Figure 3. In these experiments, 0.38% NaCl was considered to be isotonic and the calculations of the osmotic behavior were made by assuming 0.38% NaCl is equal to one unit of osmotic pressure and the volume of the eggs in this solution is equal to 1.00. The water used in these experiments was triply distilled from glass, and consequently its osmotic pressure was zero and it was not possible to use these values in the calculations. The majority of the points shown in Figure 3 yielded values of *b* between

60 and 80%. That is to say, the volume changes are in accord with the idea that these eggs are behaving as osmometers with a rather large osmotically inert volume. Similar calculations were also made assuming that 0.60% (in the absence of data in 0.66%) NaCl was isotonic with the eggs. In this case the *b*-values were 90% or larger for the majority of the points. Although this proves nothing, it does suggest that if the eggs are osmometers, more reasonable values of the amount of osmotically inactive material are obtained when a solution with a smaller osmotic pressure is assumed to be be isotonic with them.

Hyla—fertilized eggs with jelly

In this series of experiments the eggs were laid in tap water. They were subsequently removed from the jelly mass, but the layers of jelly immediately surrounding the eggs were not removed, and the eggs were then placed in the

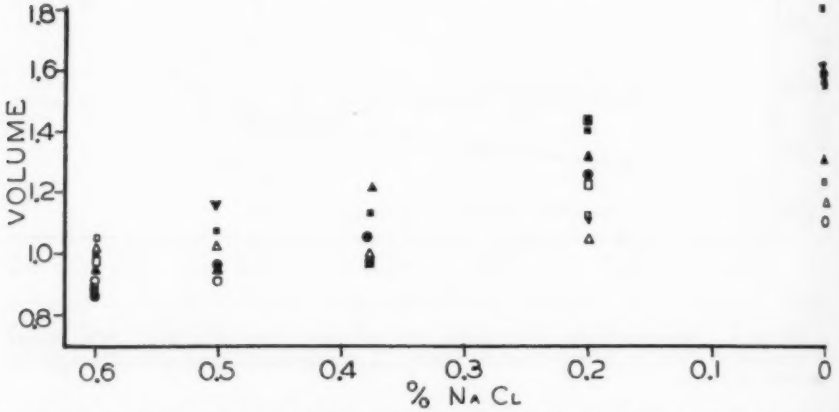

FIGURE 4. Volume changes of fertilized *Hyla* eggs (with jelly layer) when placed in solutions of NaCl of different concentrations. Open symbols: measurements made after 1–6 hours; closed symbols: after 24 hours.

appropriate sodium chloride solutions. Diameter measurements were made in some experiments between 1–4 hours after the eggs had been placed in the solutions, in others 3–6 hours and in others 6–8 hours. In most experiments they were again measured after 24 hours. During the early measurements the eggs were in early cleavage or in blastula. The eggs were in gastrula or neurula when the measurements were repeated 24 hours later. Typical data are presented in Figure 4. As in the case of fertilized eggs of *Bufo*, 0.38% NaCl was considered to have an osmotic pressure of 1.00 and the volume of the eggs in this solution was set equal to 1.00. Once again, it was not possible to use the values in water for these calculations, but for all of the other points shown in Figure 4 these eggs were behaving as osmometers with a large percentage of osmotically inactive material.

It was generally observed that the rate of development was more rapid in the

more hypotonic solutions and also the survival rate was higher (*cf.* Holtfreter, 1943). The data in the next section confirm these observations.

Hyla—fertilized eggs without jelly

In the last series of these preliminary studies, fertilized eggs of *Hyla* with the jelly removed were used. The data obtained from a single experiment are presented in Figure 5. The eggs were measured 20 minutes to an hour and a quarter after having been placed in the solutions, after 18–19 hours and again after 48 hours. The first two series of measurements were essentially the same but after 48 hours the volume changes in the two most hypotonic solutions were much

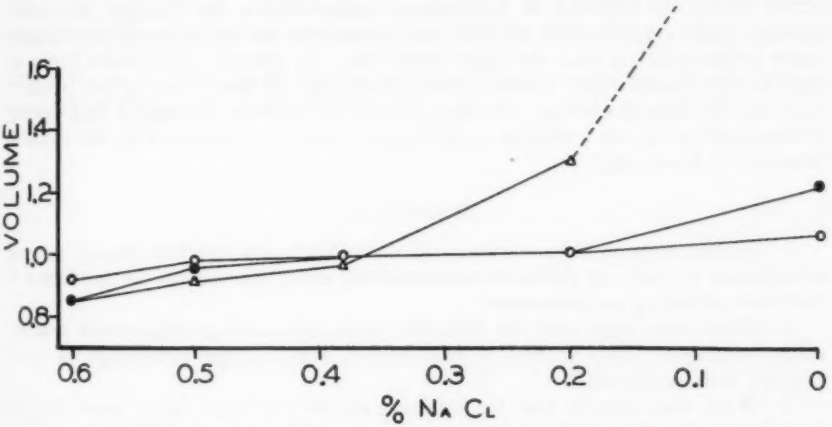

FIGURE 5. Volume changes of fertilized *Hyla* eggs (with jelly removed) when placed in solutions of NaCl of different concentrations. ○, measurements made after ⅛–1¼ hours; ●, after 18–19 hours; △, after 48 hours.

larger than after shorter exposures to these solutions. These eggs, like those with jelly, changed volumes as if they were osmometers with a large percentage of their volume osmotically inactive. At the end of 4 days all of these eggs in 0.6%, in 0.5% and in 0.38% NaCl were dead. In 0.2% NaCl 14% of the eggs were dead after 4 days and 36% after 5 days. After 5 days in water, triply distilled from glass, all of the eggs were living. In another series of observations after 4 days all of the eggs had died in 0.6% NaCl; 64% in 0.5% Nacl, 18% in 0.38% NaCl and all were living in water.

DISCUSSION

The present series of experiments was an initial preliminary survey to determine whether or not the eggs of *Bufo* and *Hyla* would change volumes in solutions of different osmotic pressures. Having shown that this is the case, these changes will subsequently be studied in more detail. Consequently, it is advisable to limit our discussion of the data at this point. However, certain generalities

seem fairly obvious. Fertilized eggs of both *Bufo* and *Hyla* change volume in solutions of NaCl of different osmotic pressures in a way that can be reconciled with the theory that they are osmometers with a relatively large osmotically inactive volume. It should be kept in mind that this is not the only explanation possible. The inclusion of the jelly layer in the volume measurements does not appreciably change the "osmotic" behavior of these cells. This might suggest that the jelly layer does not take up or give out large quantities of water in comparison with the eggs themselves. Ovarian eggs separated from the follicular layers behave in a similar manner. That is to say, their volume changes in solutions of NaCl of different osmotic pressures are in accord with the idea that they change volumes as osmometers with a large osmotically inactive volume. When the follicular layers are included in the volume measurements, the changes are much greater. This suggests that the follicular layers take up and give off much more water proportionately than the eggs themselves. In general, all of these types of eggs do not change their volumes much more after 24 hours in a given solution than they do after 2–4 hours. To date, there is no evidence to suggest that during development up to and including neurula stage, there is a difference in the osmotic behavior of these eggs.

Conclusions

1. Ovarian eggs of *Bufo marinus* with follicular layers attached change volume in solutions of NaCl of different concentrations more than would be predicted if they were behaving as osmometers.

2. These same eggs with the follicular layers removed undergo much smaller volume changes. They behave as osmometers with a large percentage of their volumes osmotically inactive.

3. These data suggest that the follicular layers exchange water more readily than do the ovarian eggs.

4. Fertilized eggs of *Bufo marinus* with jelly attached change volume as osmometers with a large percentage of their volumes osmotically inactive.

5. Fertilized eggs of *Hyla labialis* with and without jelly layer also behave as osmometers with a large percentage of their volumes osmotically inactive.

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OSMOTIC STUDIES OF AMPHIBIAN EGGS. II. OVARIAN EGGS

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In the first paper of this series (Luque and Hunter, 1959) it was demonstrated that ovarian and fertilized eggs of *Bufo marinus* and fertilized eggs of *Hyla labialis* undergo volume changes when placed in NaCl solutions of different concentrations. In the present investigation these changes in the ovarian eggs of both *Bufo* and *Hyla* were analyzed in more detail. It is hoped that this type of study may help to elucidate the mechanism of osmoregulation in these eggs and perhaps help to explain the apparent change in the osmotic pressure between the ovarian and fertilized eggs.

Materials and Methods

In order to obtain a better understanding of the volume changes previously observed, the quantity of water, sodium and potassium was first determined on the eggs removed directly from the ovaries of *Bufo* and *Hyla*. The content of these three substances was then determined when these eggs were placed in solutions of NaCl of different concentrations and finally when the eggs were placed in solutions of KCl. The eggs were removed from the ovaries with the follicular layers, and volume measurements were made using an ocular micrometer.

The water content was determined by placing a known number (usually 20–50) of eggs dried on filter paper into small, weighed procelain crucibles. The wet weight was taken immediately and the crucible was then placed in an oven at approximately 100° C. until constant weight was attained. Usually 24 hours were sufficient to reach constant weight. Attempts were also made to determine what per cent of this total water was acting as a solvent. This point seemed of considerable interest because a change in the "binding" of some of the water by cell proteins or in other ways might be an important mechanism in the osmoregulation of these eggs. The distribution method previously used by various authors (see, for example, Parpart and Shull, 1935; Hunter, 1953) was tried, using either glycerol or ethylene glycol. Unfortunately, after many attempts it was finally decided that this method was not satisfactory with these eggs. In every case a quantity of water larger than the total water of the cells was calculated by this method. It is possible that the correction applied for oxidizable material which diffused from the eggs was so large in comparison with the quantity of glycerol or ethylene glycol lost from the solution that the data were unreliable. It is hoped that this problem can be studied in the future using labelled compounds in place of the oxidation-reduction titration.

Sodium and potassium were determined using a Perkin-Elmer flame-photometer with lithium as an internal standard. Triple-glass-distilled water was used in

[1] The authors are indebted to the Rockefeller Foundation for a grant-in-aid.

every step of these experiments and all of the glassware was cleaned only in bichromate cleaning solution, distilled water and triply distilled water. The remainder of the details of the methods used will be described with a discussion of the results obtained.

Results

Ovarian eggs—Bufo

Before beginning with a study of the changes in Na and K in ovarian eggs placed in different solutions, it was necessary to determine the best method to prepare the eggs before making the salt analyses. The following three methods were tried. (1) The eggs were removed from the ovary and placed directly in a glass tube graduated at 1 cc. The volume was made up to the 1 cc. with triply distilled water and the eggs and water were transferred to a homogenizer. (2) The eggs were separated in 0.66% NaCl, then centrifuged to remove the salt solution, and then placed in the tube graduated to 1 cc. (3) The eggs were separated from the ovary in distilled water, blotted dry on filter paper and then placed in the graduated tube. Fifty eggs were used in every case.

The first method had the possible disadvantage that the ovary might dry out while the eggs were being removed, and so make the eggs abnormal. The second two methods eliminated this problem but added the complication that salts and/or water might exchange between the cells and the bathing solution, or that some of the solution might adhere to the eggs and so change the value of the their salt content.

With each method, once the tube had been filled to the 1 cc. mark with eggs and water, the contents were transferred to a glass homogenizer (a TenBroeck or a Teflon). Ten cc. of triply distilled water containing 154 ppm. of Li were added and the cells were homogenized. One cc. of this homogenate was removed for dry weight determinations (24 hours at 100° C.). The remainder of the homogenate was transferred to a 50-cc. centrifuge tube and 4 cc. of 20% tricloroacetic acid were added. After waiting for at least 15 minutes, the tube was centrifuged (2500 × G) and the supernatant fluid was used for analyses in the flame photometer.

The results of this analysis are shown in Table I. Each value was obtained from a homogenate of 50 ovarian eggs. For example, with toad #2, four separate homogenates were prepared from 200 eggs separated in 0.66% NaCl and 8 homogenates from 400 eggs separated directly from the ovary, 4 separate homogenates from 200 eggs separated in water. The individual data are included in the table to indicate the variability in analyses of different eggs from the same ovary and from different ovaries. The standard errors are included with the averages. The value of t was calculated for the difference between the value for sodium of 6.8×10^{-5} meq. per mg. dry weight and the value of potassium of 8.3×10^{-5} meq. per mg. dry weight obtained by method 1. This difference is highly significant and so it can be concluded that these eggs contain more K than Na. The difference between the two values for K obtained by methods 2 and 3 was not significant but the difference between each of these two values (4.5 and 5.7) and the value for K obtained by the first method (8.3) was highly significant. On the basis of this analysis it was concluded that when these eggs were separated in 0.66% NaCl or in water they lost K, and consequently the first method yielded the most reliable

data. The Na data did not seem to be appreciably affected by the method. In all of the remaining experiments to be described, the eggs were separated from the ovary of the female and placed directly into the experimental solutions.

TABLE I

The sodium and potassium content of ovarian eggs of Bufo marinus as measured by three different techniques (for details, see text)

Bufo No.	meq./mg. × 10^5					
	Method 1		Method 2		Method 3	
	Na	K	Na	K	Na	K
1	8.4	9.2	5.4	8.6	7.0	7.2
	8.9	9.2	6.1	4.7	8.9	8.9
					8.3	9.5
					8.4	8.4
2	6.9	8.4	7.7	4.1	7.1	3.4
	8.6	8.3	3.7	4.1	6.7	2.9
	5.7	4.5	9.1	2.3	7.2	1.5
	7.5	7.9	9.9	3.4	5.6	1.7
					9.1	2.9
					4.7	2.8
					8.2	1.8
					6.4	2.1
3	7.0	8.5			5.4	7.7
	8.5	8.7			3.2	6.5
	6.8	7.8			6.2	8.9
	9.5	8.5			5.5	7.7
4	8.4	8.8			4.1	6.9
	7.1	9.3			6.3	7.8
	6.8	9.6			5.5	8.2
	5.7	9.4			8.8	7.3
5	6.5	9.6				
	6.6	9.2				
	6.6	8.8				
	4.6	8.4				
	7.5	7.8				
	5.7	6.3				
	4.0	6.7				
	4.5	6.6				
	4.6	7.7				
	8.9	9.3				
Averages	6.8 ± 0.32	8.3 ± 0.25	7.0 ± 0.92	4.5 ± 0.81	6.6 ± 0.36	5.7 ± 0.66

The water content of "normal" ovarian eggs of *Bufo* was determined on three different females. Twenty to 50 eggs with their follicular layers were removed from the ovary, blotted on filter paper and placed in a crucible and weighed immediately and after 24 hours at 100° C. The data are presented in Table II.

TABLE II

The water content of ovarian eggs of Bufo marinus

Bufo no.	No. of weighings	% water	mg. water/cell
1	17	55 ± 0.4	
2	8	56 ± 1.2	0.58 ± 0.03
3	10	48 ± 1.5	0.70 ± 0.03

As can be seen, not only is there considerable difference between the water content of eggs from different ovaries but also considerable variability with eggs from the same ovary.

Ovarian eggs—Bufo—NaCl solutions

Having determined the values for eggs removed directly from the ovary, the effect of placing the eggs in various NaCl solutions was next studied. Since volume measurements had previously been made (see Figure 1 of preceding paper), they were not repeated in this series of experiments. Eggs were removed from the ovary and placed in the various solutions of NaCl in covered dishes. After two or more hours, 20–30 eggs were removed, blotted on filter paper, weighed and dried for wet and dry weights. Fifty additional eggs were dried on filter paper and placed in the tube calibrated to 1 cc. for the salt determinations. A second homogenate was prepared with another 50 eggs for duplicate determinations. The data obtained from 5 experiments are presented in Tables III and IV. It can be seen from Table III that the changes in per cent water and milligrams of water per egg roughly parallel the changes in volume previously reported. The average relative values are also included (each value divided by the value in 0.66% NaCl). From a comparison between Table I and Table IV it can be seen that in all of the solutions there is a significant increase in the Na content of the eggs but the amount of K does not change appreciably.

TABLE III

Changes in water content of ovarian eggs of Bufo marinus placed in solutions of NaCl of different osmotic pressures

Bufo no.	Control		0.75% NaCl		0.70% NaCl		0.66% NaCl		0.60% NaCl		0.55% NaCl	
	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg
1	—	—	72	1.34	63	0.90	65	1.09	68	1.09	77	1.83
2	—	—	64	1.28	66	1.61	66	1.55	65	1.43	71	2.12
3	49	0.60	45	0.61	57	1.02	58	1.05	58	1.01	65	1.41
4	51	0.72	56	1.09	53	0.83	59	1.04	64	1.20	60	1.00
5	43	0.78	55	1.04	49	0.90	56	1.12	54	0.98	59	1.25
Average	48	0.70	58	1.07	57	1.05	61	1.17	62	1.14	66	1.52
Average (relative)			0.95	0.91	0.93	0.90	1.00	1.00	1.02	0.97	1.08	1.30

TABLE IV

Changes in salt content (meq./mg. × 10^5) of ovarian eggs of Bufo marinus placed in solutions of NaCl of different osmotic pressures

Bufo no.	0.75% NaCl		0.70% NaCl		0.66% NaCl		0.60% NaCl		0.55% NaCl	
	Na	K	Na	K	Na	K	Na	K	Na	K
1	14.5	6.3	17.4	7.6	15.0	8.2	14.8	9.2	14.2	9.1
2	16.6	8.8	15.5	7.8	15.8	9.1	16.3	8.6	13.0	7.6
3	13.0	8.5	12.5	7.4	11.2	6.4	12.9	9.8	7.4	6.1
4	13.0	8.0	12.8	7.7	12.0	8.1	16.8	8.4	11.3	7.8
5	7.1	6.9	6.9	6.5	7.5	7.2	6.9	6.4	6.5	7.6
Average	12.9	7.7	13.1	7.4	12.2	7.8	13.5	8.4	10.5	7.6

Ovarian eggs—Bufo—KCl solutions

A similar series of observations were then made, placing ovarian eggs of *Bufo* in solutions of KCl. As before, the eggs were removed from the ovary and placed in solutions of KCl in covered dishes. After two or more hours, the diameters of 10 eggs were measured, 20 eggs were selected for weighing and 50 eggs for salt determinations. Figure 1 shows that the volume changes were small with *b*-values positive ranging from 0 to about 0.80 (the same scale has been used with these figures as was used in the preceding paper). The data for water content (Table V) show changes similar to the volume changes. Table VI shows that in all of the solutions, Na leaves the cells and K enters.

Ovarian eggs—Hyla

Measurements were made using ovarian eggs of *Hyla* (with follicular layers attached) from three females, to determine the "normal" content of water, Na and K in these eggs. Three or four groups of 25 eggs were placed in crucibles

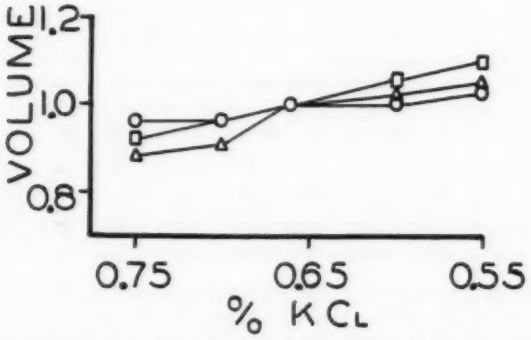

FIGURE 1. Volume changes of ovarian eggs of *Bufo* (with follicular layers) when placed in solutions of KCl of different concentrations. Measurements made after two or more hours.

TABLE V

Changes in water content of ovarian eggs of Bufo marinus placed in solutions of KCl of different osmotic pressures

Bufo no.	Control		0.75% KCl		0.70% KCl		0.66% KCl		0.60% KCl		0.55% KCl	
	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg
1	50	0.50	52	0.77	61	0.79	61	0.87	62	0.81	64	0.91
2	48	0.57	66	1.15	70	1.21	70	1.31	72	1.42	74	1.48
3	41	0.35	47	0.40	63	0.63	61	0.61	62	0.71	65	0.83
Average	46	0.47	55	0.77	65	0.88	64	0.93	65	0.98	68	1.07
Average (relative)			0.86	0.83	1.02	0.95	1.00	1.00	1.02	1.05	1.06	1.15

for wet and dry weight determinations and three or four groups of 50 eggs were used for salt determinations. The values for per cent of water, quantity of water per egg and milliequivalents of Na and K per milligram dry weight are shown in Table VII. These eggs contain slightly more water than those of *Bufo* but less sodium and potassium. There is less difference in these *Hyla* eggs between the amount of Na and K than in the *Bufo* eggs but there is significantly more K in both types of eggs.

Ovarian eggs—Hyla—NaCl solutions

Similar observations were then made using *Hyla* ovarian eggs in solutions of NaCl. Because of the smaller number of eggs in each ovary, the 10 eggs used for diameter measurements were subsequently added to 40 other eggs for salt determinations and only one homogenate was prepared. Twenty eggs were used for wet and dry weights. These data are presented in Figure 2 and in Tables VIII and IX. All of the changes observed are very similar to those obtained with *Bufo*. The volume measurements were made after one or two hours' exposure to the solutions and the observed changes (Fig. 2) are small. The largest changes are slightly greater than would be predicted if these eggs were osmometers

TABLE VI

Changes in salt content (meq./mg. $\times 10^5$) of ovarian eggs of Bufo marinus placed in solutions of KCl of different osmotic pressures

Bufo no.	0.75% KCl		0.70% KCl		0.66% KCl		0.60% KCl		0.55% KCl	
	Na	K	Na	K	Na	K	Na	K	Na	K
1	4.5	11.8	3.0	10.8	3.8	9.2	2.6	10.6	3.2	8.8
2	6.8	15.1	4.7	12.3	1.5	11.7	7.0	9.1	9.2	9.0
3	3.1	8.1	3.3	20.1	2.4	17.7	2.0	14.7	1.2	15.2
Average	4.8	11.7	3.7	14.4	2.6	12.9	3.9	11.5	4.5	11.0

TABLE VII

The content of water, Na and K in ovarian eggs of Hyla labialis

Hyla no.	No. of weights	% water	mg. water/egg	meq./mg. × 10^5	
				Na	K
1	3	64 ± 0.57	1.02 ± 0.039	4.3	4.6
				5.0	5.5
				5.5	5.5
2	4	49 ± 0.83	0.72 ± 0.029	3.6	5.4
				3.8	5.7
				3.6	5.2
				3.6	5.2
3	4	52 ± 0.50	0.65 ± 0.024	5.1	5.6
				5.1	5.6
				4.5	5.4
				4.8	5.3
Averages		55	0.80	4.4 ± 0.20	5.4 ± 0.028

(negative *b*-values) but the majority of the differences yield positive values of *b*. The changes in water content (Table VIII) roughly parallel the volume changes. These cells gain a considerable quantity of Na and lose a small amount of K (Table IX).

Ovarian eggs—Hyla—KCl solutions

The last series of measurements were made using ovarian eggs of *Hyla* in solutions of KCl after a one-hour exposure to these solutions. These data are

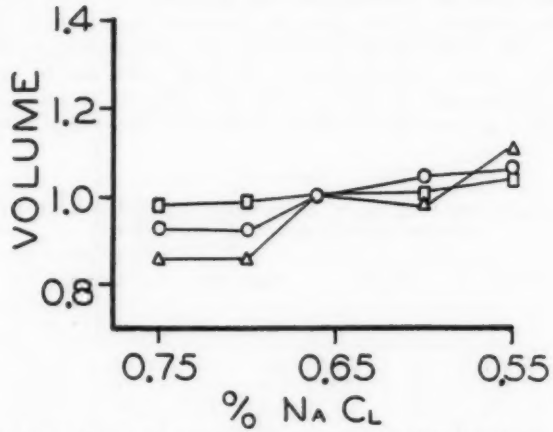

FIGURE 2. Volume changes of ovarian eggs of *Hyla* (with follicular layers) when placed in solutions of NaCl of different concentrations. Measurements made after one or two hours.

TABLE VIII

Changes in water content of ovarian eggs of Hyla labialis placed in solutions of NaCl of different osmotic pressures

Hyla no.	Control		0.75% NaCl		0.70% NaCl		0.66% NaCl		0.60% NaCl		0.55% NaCl	
	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg
1	47	0.46	45	0.39	48	0.51	48	0.52	48	0.49	48	0.46
2	64	0.80	56	0.50	56	0.62	61	0.64	60	0.63	61	0.64
3	50	0.66	45	0.55	47	0.60	51	0.76	50	0.72	59	1.03
Average	54	0.64	49	0.48	50	0.58	53	0.64	53	0.61	56	0.71
Average (relative)			0.92	0.75	0.94	0.91	1.00	1.00	1.00	0.96	1.06	1.11

presented in Figure 3 and in Tables X and XI. As before, the largest volume changes give negative values of b but the majority of changes could be explained on the basis of the cells being osmometers. Once again, the changes in water content are similar to the volume changes (Table X). The quantity of Na in these eggs does not change appreciably but there is a large increase in the amount of K (Table XI).

DISCUSSION

The errors involved in a study of this type make it difficult to draw strictly quantitative conclusions. For example, from one toad or from one frog to another, particularly when using species which are not exposed to marked seasonal changes, the size of the largest eggs in the ovary will vary considerably. The eggs which showed the largest volume changes in solutions of NaCl, in the study of *Bufo*, had an average volume of 0.542 mm.^{3} in 0.66% NaCl while the eggs which showed much smaller volume changes had an average volume of 2.580 mm.^{3}. If the conclusion is correct that the follicular layers change volume proportionally much more than the eggs (*cf.* Luque and Hunter, 1959), this might explain the larger volume changes with the smaller eggs. But since it is obviously impossible

TABLE IX

Changes in salt content (meq./mg. $\times 10^3$) of ovarian eggs of Hyla labialis placed in solutions of NaCl of different osmotic pressures

Hyla no.	0.75% NaCl		0.70% NaCl		0.66% NaCl		0.60% NaCl		0.55% NaCl	
	Na	K	Na	K	Na	K	Na	K	Na	K
1	4.8	3.3	6.1	3.0	5.0	3.4	7.0	3.5	6.3	2.9
2	9.2	3.6	8.4	3.9	7.2	3.7	8.6	3.4	7.1	3.8
3	11.0	4.7	10.0	3.0	9.3	2.3	9.2	2.3	13.0	2.8
Average	8.3	3.9	8.2	3.3	7.2	3.1	8.3	3.1	8.8	3.2

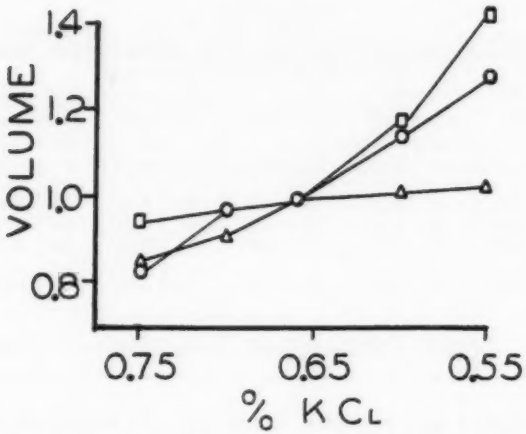

FIGURE 3. Volume changes of ovarian eggs of *Hyla* (with follicular layers) when placed in solutions of KCl of different concentrations. Measurements made after one hour.

to study all of the aspects of a problem such as this at one time, it seems best to use the material, heterogeneous as it may be, and see what general information can first be obtained.

Ovarian eggs of *Bufo* (with follicular layers) have a water content of 50 ± 10%, 6.8×10^{-5} milliequivalents of Na per mg. dry weight and 8.3×10^{-5} milliequivalents of K per mg. dry weight. From Table II, using the data from *Bufo* 2 and 3 we obtain the values of 52% water and 0.64 mg. of water per egg. The dry weight of each egg, then, is 0.59 mg. Multiplying this weight by the values for Na and K we see that each egg contains 4.0×10^{-5} milliequivalents of Na and 4.9×10^{-5} milliequivalents of K. Knowing the quantity of water in each egg we can calculate the sum of the milliequivalents of these two cations in one liter of egg water. With the above data, this has the value of 140. That is to say, con-

TABLE X

Changes in water content of ovarian eggs of Hyla labialis placed in solutions of KCl of different osmotic pressures

Hyla no.	Control		0.75% KCl		0.70% KCl		0.66% KCl		0.60% KCl		0.55% KCl	
	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg
1	48	0.61	60	1.01	60	1.03	60	1.04	60	1.06	63	1.26
2	48	0.69	69	1.47	70	1.84	70	1.68	73	1.51	73	1.53
3	58	0.85	70	1.43	73	1.67	73	1.68	73	1.83	74	1.86
Average	51	0.72	66	1.30	68	1.51	68	1.47	69	1.47	70	1.55
Average (relative)			0.97	0.88	1.00	1.03	1.00	1.00	1.02	1.00	1.03	1.06

TABLE XI

Changes in salt content (meq./mg. × 10^5) of ovarian eggs of Hyla labialis placed in solutions of KCl of different osmotic pressures

Hyla no.	0.75% KCl		0.70% KCl		0.66% KCl		0.60% KCl		0.55% KCl	
	Na	K	Na	K	Na	K	Na	K	Na	K
1	3.2	11.4	3.7	13.2	5.6	11.8	3.5	11.5	3.2	12.9
2	5.6	19.0	4.6	14.5	7.9	19.4	3.7	18.0	5.9	17.0
3	4.0	17.4	3.1	17.4	3.9	16.0	2.5	14.8	2.5	15.2
Average	4.3	15.9	3.8	15.0	5.8	15.7	3.2	14.8	3.9	15.0

sidering only Na and K, these eggs have a salt content equivalent to 0.140 *M* NaCl. This value seems a little high but at least it suggests that two assumptions are probably quite reasonable: (1) that Na and K constitute the major quantity of cations in these eggs and (2) 0.66% NaCl should have an osmotic pressure much closer to that of the eggs than 0.38% NaCl.

When *Bufo* eggs are placed in solutions of NaCl with osmotic pressures not much different from that which is assumed to be isotonic, the per cent of water and the amount of Na in the eggs increase in all of the solutions, but the amount of K does not change appreciably. In solutions of KCl of the same concentrations, the per cent of water increases approximately the same amount, the amount of K increases and Na decreases. These observations show that water, Na and K can move into and out of the eggs to a limited extent, at least. The cations did not exchange to diffusion equilibrium during the duration of these experiments. The question as to whether or not these *Bufo* eggs are behaving as osmometers is diffi-

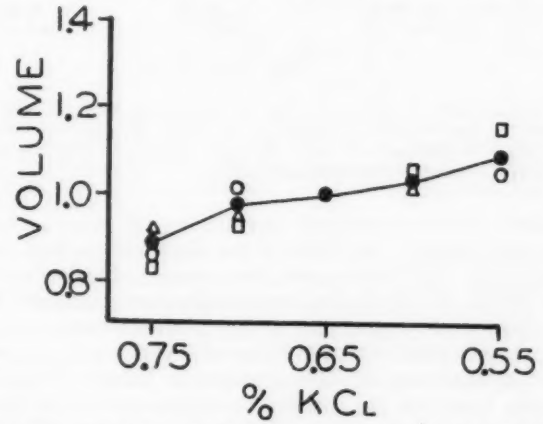

FIGURE 4. Ovarian eggs of *Bufo* (with follicular layers) when placed in solutions of KCl of different concentrations. △, volume changes; □, water content per egg; ○, % water; ●, average of the three preceding values.

cult to answer because of the variability of the data and the errors involved in the measurements. In general, however, the majority of the data are not inconsistent with the hypothesis that these *Bufo* eggs are changing volume as osmometers with a relatively large proportion of their volumes osmotically inactive. Since the dry weight of these eggs is equal to 50 ± 10% of the volume, the *b*-value would have to be approximately 0.5 if all of the cell water were osmotically active. Three different values have been used to try to measure volume changes: (1) diameter measurements; (2) % water; and (3) quantity of water per egg. The last two values depend on wet and dry weights but the amount of water per cell takes into account differences in the size of the eggs. In general, when these three sets of

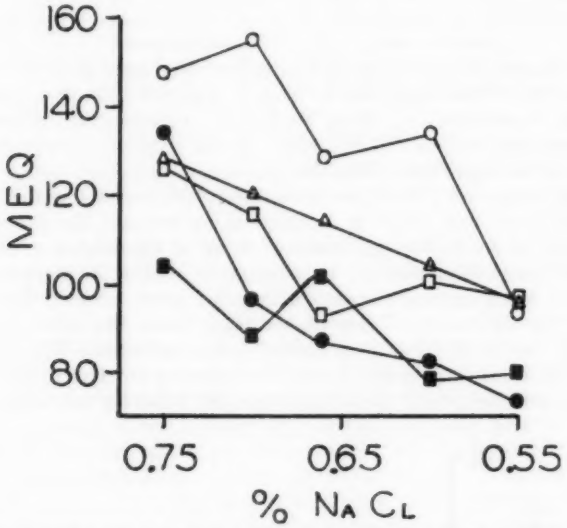

FIGURE 5. Milliequivalents of cations per liter of ovarian egg water in solutions of different salt concentrations. ○, eggs of *Bufo* in solutions of NaCl; ●, eggs of *Bufo* in solutions of KCl; □, eggs of *Hyla* in solutions of NaCl; ■, eggs of *Hyla* in solutions of KCl; △, calculated values (% NaCl converted to milliequivalents).

data are calculated as relative changes (considering the value in 0.66% NaCl as 1.00) they are very similar. In Figure 4 are plotted these data for the eggs of *Bufo* in KCl solutions. The line connects the averages of these three values. The four points for 0.70% to 0.55% NaCl essentially form a straight line with a *b*-value of 0.52. This would suggest that in these four solutions, at least, these cells were behaving as osmometers with all of the cell water being osmotically active.

Another way of analyzing the data is shown in Figures 5 and 6. Using the method previously described, the number of milliequivalents of Na plus K in a liter of cell water was calculated from the values in Tables III, IV, V and VI (Tables VIII, IX, X and XI for *Hyla*). The straight line (Fig. 5) merely represents the milliequivalents of Na in each of the 5 solutions. If the quantity of

water and/or cations is changing as a consequence of the change in the amount of cation in the surrounding solution (*i.e.*, osmotic pressure), one would expect that these eggs would have a smaller concentration of cations when placed in the less concentrated salt solutions. This might result either from the entrance of water or the exit of cations. If the eggs were behaving as osmometers, the curve relating the concentration of cations within the eggs with the external salt concentration would be parallel with the theoretical curve. On the other hand, if the eggs did not gain or lose water and/or cations, these experimental curves should be parallel

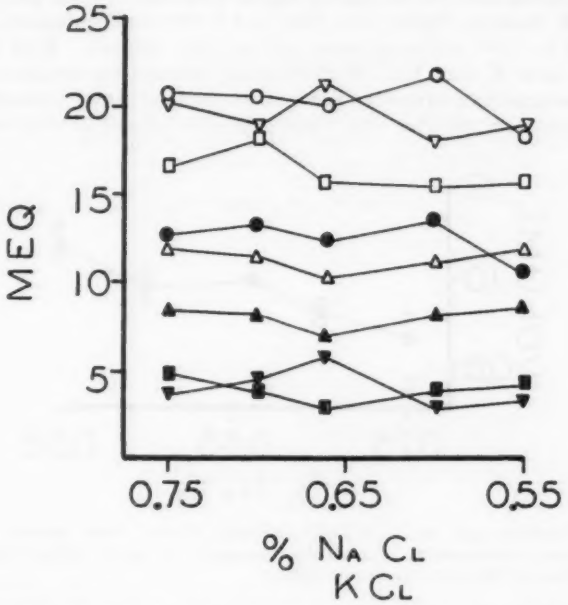

FIGURE 6. Milliequilivants of cations ($\times 10^5$) per ovarian egg in solutions of different salt concentrations. ○, sum of Na and K—*Bufo* eggs in NaCl; ●, Na—*Bufo* eggs in NaCl; ▽, sum of Na and K—*Hyla* eggs in KCl; ▼, Na—*Hyla* eggs in KCl; □, sum of Na and K—*Bufo* eggs in KCl; ■, Na—*Bufo* eggs in KCl; △, sum of Na and K—*Hyla* eggs in NaCl; ▲, Na—*Hyla* eggs in NaCl.

with the x-axis. Although the curves in Figure 5 are far from smooth, their slopes much more nearly parallel the theoretical straight line than the x-axis. Figure 6 shows that it is the quantity of water that is changing and not the total quantity of cations or of Na or K. These curves in Figure 6 are roughly parallel with the x-axis. The curves with the open symbols show the sum of Na plus K in solutions of NaCl and KCl while the curves with the closed symbols show the Na content in these two sets of solutions. Considering the *Bufo* eggs, the sum of Na and K, as well as their concentrations, is higher in the NaCl solutions than in KCl. This might result from the apparent difference in the net exchange of Na and K. In solutions of NaCl more sodium appears to enter the cells than K enters in

solutions of KCl. Furthermore, more Na leaves the cells in solutions of KCl than K leaves the cells in solutions of NaCl. As a consequence of this apparent more ready exchange of Na than K, the eggs in NaCl solutions contain more cations. The salt osmotic pressure in the eggs in solutions of NaCl appears to be greater than that of the surrounding solutions, whereas in KCl solutions, it is less. Whether this is a rate or equilibrium difference cannot be determined by these studies.

Turning now to a similar analysis of the data obtained with *Hyla* ovarian eggs, their water content seems to be slightly higher than that of *Bufo* ($55 \pm 10\%$), and the Na and K contents slightly less (Na: 4.4×10^{-5} milliequivalents per mg. dry weight; K: 5.4×10^{-5} milliequivalents per mg. dry weight). Both types of eggs have slightly more K than Na. Without going through the details a second time, the data on volume measurements, per cent water, and water content per egg are not inconsistent with the idea that these eggs also behave as osmometers, within

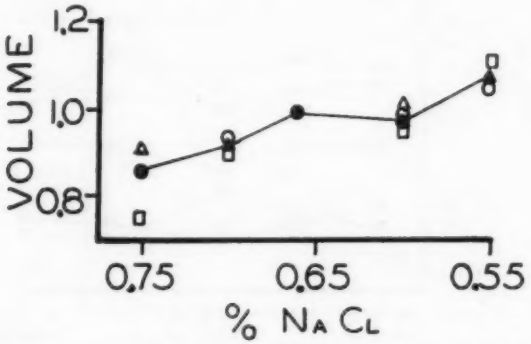

FIGURE 7. Ovarian eggs of *Hyla* (with follicular layers) when placed in solutions of NaCl of different concentrations. △, volume changes; □, water content per egg; ○, % water; ●, average of the three preceding values.

the limited range of solutions used, with all of the water being osmotically active. Figure 7 compares with Figure 4. The data for *Hyla* are also included in Figures 5 and 6. From Figure 5 it can be seen that in solutions of NaCl the cation concentrations within the eggs are essentially equal to the concentration in the surrounding solutions. This would suggest that the osmotic pressure of *Hyla* eggs is somewhat less than that of *Bufo* eggs. Once again, the concentrations of the cations are less in the KCl solutions than in the NaCl solutions but the quantity of cations was greater in the cells in the KCl solutions, resulting from the rather large amount of K which entered into the eggs.

CONCLUSIONS

1. Ovarian eggs of *Bufo marinus* (with follicular layers attached) contain $50 \pm 10\%$ water, 6.8×10^{-5} milliequivalents of Na per mg. dry weight and 8.3×10^{-5} milliequivalents of K per mg. dry weight.

2. The data suggest that when these eggs are placed in solutions of NaCl or KCl of different concentrations (0.55–0.75%), they change volumes as osmometers with all of the water acting as a solvent.

3. Ovarian eggs of *Hyla labialis* (with follicular layers attached) contain $55 \pm 10\%$ water, 4.4×10^{-5} milliequivalents of Na per mg. dry weight and 5.4×10^{-5} milliequivalents of K per mg. dry weight.

4. The data also suggest that these eggs in solutions of NaCl and KCl change volumes as osmometers with all of the water acting as a solvent.

5. Water, Na and K can move across the membranes of both *Bufo* and *Hyla* eggs.

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STUDIES ON THE ACTION OF PHENYLTHIOUREA ON THE RESPIRATORY METABOLISM AND SPINNING BEHAVIOUR OF THE CYNTHIA SILKWORM

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There is now abundant proof, furnished particularly by Schneiderman and Williams (1954), and Shappirio and Williams (1957a, 1957b), of the presence of various cytochromes and of cytochrome oxidase in the tissues of the *Cecropia* silkworm.

In the larval stage, the cytochrome system is both intact and functional, and sensitive to cyanide and carbon monoxide. During the course of metamorphosis, however, there is a precipitous fall in the rate of oxygen consumption of the *Cecropia* silkworm, and, later, when diapause intervenes during the pupal stage, the presence of cytochromes *b* and *c* becomes indetectable, while cytochrome b^5 and cytochrome oxidase are present at low levels according to spectrophotometric and spectroscopic studies of individual tissues. These changes do not occur in the cytochrome system of the intersegmental muscles of the abdomen (Shappirio and Williams, 1957a, 1957b). The partial breakdown of the cytochrome system in the rest of the tissues, however, seems to be responsible for the striking decrease in respiratory metabolism.

A distinguishing feature of the metabolism of the diapause pupa is its resistance to cyanide and carbon monoxide. This is apparently due to the great excess of cytochrome oxidase relative to cytochrome *c* in this stage, and to cyanide and carbon monoxide being incapable of inhibiting this reserve cytochrome oxidase unless the oxygen tension is experimentally decreased to exceedingly low levels (Harvey and Williams, 1958a, 1958b; Kurland and Schneiderman, 1959). Previously, the cyanide- and carbon monoxide-insensitive character of the metabolism of the diapause pupa suggested the possibility that tyrosinase might be serving as a terminal oxidase in respiration instead of cytochrome oxidase. Sussman (1949, 1952), however, showed that this was not the case.

Since it has long ago been shown that cytochrome oxidase is immune to the action of various urea compounds (Grant and Krantz, 1942) and phenylthiourea (DuBois and Erway, 1946), it has been customary to use the latter compound, a copper-catalyst blocking agent, to inhibit the activity of blood phenolase to prevent the formation of toxic quinolic substances after surgical operation on insects. It was therefore of particular interest that the use of the compound for this purpose in some experiments on Cynthia silkworms seemed to depress their metabolism.

Preliminary studies on the oxygen consumption of *Cynthia* revealed significant decreases in the rate after treatment with phenylthiourea. Moreover, it was also noticed that treatment with this compound affected the nature of the cocoon spun later by the silkworm.

The difference in degree of respiratory metabolism, as shown by these preliminary results, suggested that the respiratory metabolism of *Cynthia*, though probably mediated by the usual cyanide- and carbon monoxide-sensitive cytochrome oxidase system, is sensitive to the action of a copper-catalyst inhibitor.

This investigation was therefore undertaken to elucidate further the depressant effect of phenylthiourea on respiratory metabolism, and the accompanying effect of this compound on the spinning behaviour of *Cynthia*.

Materials and Methods

The present study is based on respiratory measurements of final instar larvae and pupae of the silkworm, *Philosamia cynthia*.

Phenylthiourea was introduced into the blood of the silkworm, under CO_2 anaesthesia, either by inserting it as crystals through an incision made in the abdomen, or by injecting it in a physiological solution from a hypodermic syringe.

Both the experimental and control animals were kept in glass tubes containing a strip of moistened filter paper, and were fed on privet. They were transferred to vessels of approximately 40-cc. for use with standard Warburg manometers for oxygen-consumption measurements. Each vessel was divided into two connected well-compartments by an infolding of the base. To absorb the carbon dioxide output, a loose roll of filter paper was moistened with 0.5 to 0.7 cc. of 1.5 N sodium hydroxide and placed in one of the compartments, the silkworm being placed in the other. To protect the silkworm, another strip of filter paper was placed over the moistened roll. The silkworms only very occasionally disturbed the arrangement of filter papers.

Measurements were performed at 25° C. They were usually carried out at intervals of ten minutes over a period of half an hour or longer. The rate has been expressed as mm.^{3} oxygen per gram weight of the pupa per hour, following the procedure of Schneiderman and Williams (1953), to obviate the changeability in weight of a feeding and spinning silkworm.

Results

1. *Normal changes in oxygen consumption during metamorphosis*

The maximal rate of oxygen consumption in *Cynthia* prior to metamorphosis is in the region of 1400 mm.^{3} O_2/gm.pupal wt./hr. at 25° C. (Fig. 1).

At metamorphosis, considerable changes take place. The rate of oxygen consumption begins to fall two and a half days before the onset of spinning. It continues to fall, reaching a level of about 250 mm.^{3} O_2/hr. at the end of spinning and of 75 mm.^{3} O_2/hr. in the pupal stage.

The precipitous nature of this fall in the rate of oxygen consumption in *Cynthia* during the course of metamorphosis at 25° C. is in agreement with that recorded for *Cecropia* (Schneiderman and Williams, 1953). There is, however, an important difference with regard to the timing of the fall. Whereas in *Cecropia* the decrease begins just after the cocoon has been spun, the decrease in *Cynthia* begins two and a half days before spinning is initiated. Indeed, at the beginning of the spinning phase, in *Cynthia*, the rate of oxygen consumption has already fallen to half the maximal level.

Cynthia takes two days to construct a cocoon, the outer envelope being completed in 24 hours, and the loose intermediate layer and inner envelope in the next 24 hours.

2. *Inhibitory effect of phenylthiourea on oxygen consumption*

Measurements of the rate of oxygen consumption were carried out on feeding fifth instar *Cynthia* before and after treatment with phenylthiourea. The compound was inserted as crystals through an incision made in the abdomen. Though

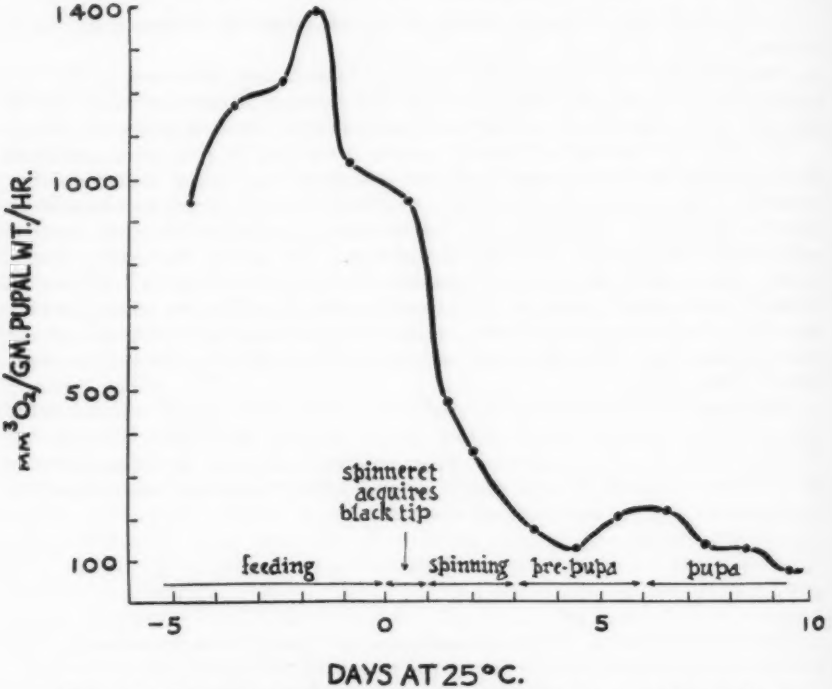

FIGURE 1. Changes in the rate of oxygen consumption at 25° C. of the *Cynthia* silkworm during metamorphosis.

it is highly insoluble in water, being taken up at about 2 mg. per cc., it dissolves readily in *Cynthia* blood. The weights of the silkworms used ranged from 3 to 4 gm.

Figure 2 shows the depressant action of the inhibitor at varying amounts on the rate of oxygen consumption. When 0.45 mg. was introduced in the way just described, there was a significant decrease in the rate of oxygen consumption of the treated silkworm. The rate subsequently increased without, however, reaching the pre-treatment level. At the onset of spinning the rate was about 650 mm.^{3} O_2/hr.

At 0.75 mg., the depressant action of the compound on the rate was more marked. The fall in the rate, however, was again followed by an increase, but this failed to reach a level above 500 mm.^{3} O_2/hr. before spinning was initiated. Moreover, silkworms treated with this amount of the compound were rendered incapable of constructing a normal cocoon, but they were not, however, prevented from pupating normally.

At 1.0 mg., there was a striking fall in the rate. Silkworms treated with this amount of the compound were, after a delay of several hours, liable to become completely immobilized. This induced quiescent state usually lasted about two

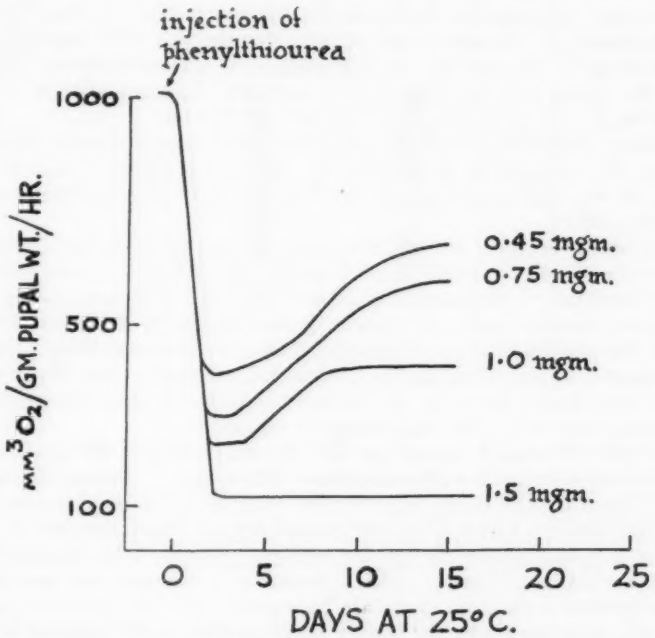

FIGURE 2. Depressant action of phenylthiourea at different concentrations on the rate of oxygen consumption of feeding fifth instar *Cynthia* larvae at 25° C.

days. This was followed by a gradual recovery which was accompanied by an increase in the rate of oxygen consumption. The rate, however, seldom reached a level above 400 mm.^{3} O_2/hr. before spinning took place. Though the usual two-day period was spent in spinning by these treated silkworms, it was observed that they failed to produce more than a limited sheet of silk.

Amounts of phenylthiourea in excess of 1.5 mg. were usually lethal to the silkworms used. They invariably brought about a precipitous fall in the respiratory rate, and so completely immobilized the treated silkworms that they seldom showed any signs of recovery.

These studies indicate that the depressant action of phenylthiourea on the rate of oxygen consumption in *Cynthia* is proportional to its concentration in the blood and tissues. When amounts of less than 0.45 mg. were introduced into the blood, they also significantly lowered the respiratory rate, and the results which indicate this are considered in a later section of this paper. Moreover, it will be apparent that the *Cynthia* silkworm seems capable of reversing the depressant action of phenylthiourea on its respiratory metabolism, provided the amount of the compound administered is less than the lethal dose.

3. *Inhibition of blood phenolase activity*

Efforts were next made to determine the inhibitory effect of phenylthiourea on the blood phenolase. Though it was unlikely that the site of the depressant action on the respiratory rate was the blood phenolase, it was nevertheless desirable to estimate the ability of this compound to inactivate a copper-catalyst which was known to be present in the blood and probably the tissues.

Phenolase (tyrosinase) is distinguished from other copper enzymes by its ability to catalyze the insertion of an hydroxyl group into monohydric phenols and the oxidation of the resulting o-diphenols to their corresponding o-quinones (Dawson and Tarpley, 1951).

It is generally accepted that the enzyme is present in the blood of insects, and that when the blood is exposed to air it changes to a brown colour owing to the enzymatic oxidation of polyphenols to form melanin-like substances. The addition of a diphenol substrate such as dihydroxyphenyl-alanine to the blood of an insect results in the rapid formation of melanin, due to the reaction being catalyzed by the phenolase. If phenylthiourea is also added, melanin is not deposited. The inference to be drawn from this is that this compound in blocking the activity of the phenolase suppresses the development of melanin.

Most inhibition studies carried out with the enzyme have been performed with substances known to form stable complexes with copper. Among the substances used, the thioureas seem to be very effective blocking agents of phenolase (DuBois and Erway, 1946). Little is known at the present time, however, as to how the copper is bound within the enzyme, or what precisely is its role in phenolase activity (Dawson and Tarpley, 1951). It is likely, however, that phenylthiourea exerts its inhibitory effect on phenolase by reacting with the copper component of this enzyme, as it does with the copper which catalyzes the oxidation of ascorbic acid (DuBois and Erway, 1946).

Dawson and Tarpley (1951) have directed attention to the fact that the oxidation of di- and polyhydric phenols can be brought about in other ways. For example, these phenols can be oxidised by hydrogen peroxide in the presence of peroxidase, and aerobically in the presence of cytochrome *c* and cytochrome oxidase. Presumably, this type of oxidation cannot be inhibited by a copper-catalyst blocking agent such as phenylthiourea which has no effect on the enzymes concerned. Copper alone, or in complex form with non-specific proteins, peptides, or amino acids, can also apparently catalyze the aerobic oxidation of these phenols. There is strong evidence, however, in support of the conclusion that in insects a blood phenolase (tyrosinase) is responsible for the formation of melanin from polyphenols (Mason, 1955).

The following tests were carried out to permit an estimation of to what extent the blood phenolase of *Cynthia* could be inhibited by the introduction of phenylthiourea. Silkworms weighing about 3 to 4 gm. were treated with amounts of either 0.45 mg. or 1 mg. of the compound by the method already described. Samples of blood were taken from these silkworms 24 hours later. To the samples was added a solution of dihydroxyphenyl-alanine so that 1 cc. of the final solution contained 1 mg. of the phenol substrate. Figure 3 illustrates samples of blood from silkworms treated or untreated previously with the inhibitor, and also samples to which the substrate has been added.

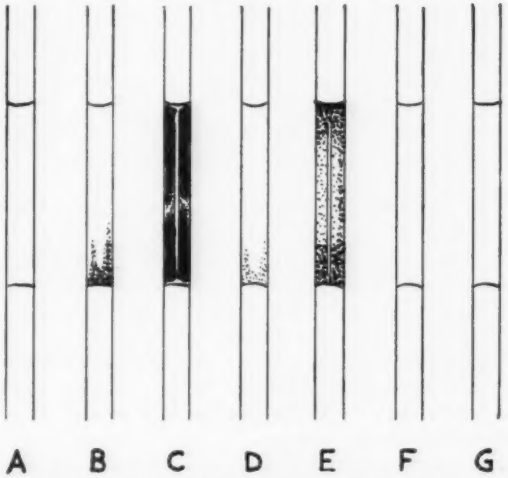

FIGURE 3. Inhibitory effect of phenylthiourea on melanin formation in *Cynthia* blood. For details see text. A, solution of diphenol substrate; B, untreated blood; C, untreated blood incubated with the diphenol; D, blood from silkworm injected 24 hours previously with 0.45 mg. of the inhibitor; E, same as D incubated with the diphenol; F, blood from silkworm injected 24 hours previously with 1 mg. of the inhibitor; G, same as F incubated with the diphenol. Drawings taken from photographs of the results of this experiment.

Though distinctions could be drawn after a few hours between the different samples according to the intensity of melanin development, they became more clearly marked at the end of 24 hours (Fig. 3). There seemed to be no further increase in the intensity of melanin deposited after about 30 hours.

It is concluded from a series of tests of the kind illustrated in Figure 3 that whereas 0.45 mg. of phenylthiourea partially blocked the activity of the blood phenolase, 1 mg. completely blocked it. Moreover, the inference to be drawn from the results of these tests is that the inhibitor is inactivating the enzyme *in vivo*.

4. *Effect of changes in the metabolic rate on cocoon spinning*

To elucidate further the effect of phenylthiourea upon the spinning behaviour of *Cynthia*, studies were next made on the depressant action of this compound on respiratory metabolism during the spinning period.

TABLE I

Relationships between the depressant action of phenylthiourea on the rate of oxygen consumption at the beginning of spinning and the types of structures spun. For details see text.

Serial no.	Mg. of agent injected 24 hr. previous to spinning	mm.³ O_2/gm. pupal wt./hr.			Types of cocoon spun
		Uptake at the beginning of spinning	Uptake at the end of spinning	Range	
P 6	nil	740	210	520	Normal
P 8	0.1	700	260	440	Two-layered and closed
P 1	0.1	700	280	420	Two-layered and closed
P 4	0.3	520	280	240	Two-layered and open
P 9	0.4	500	250	250	Hammock-shape
P 7	0.5	525	265	260	One-layered and open
L 2	0.6	490	290	200	Hammock-shape
P 2	0.7	385	210	175	Hammock-shape

An important symptom of metamorphosis in *Cynthia*, as already described, is the striking fall in the rate of oxygen consumption, which begins two and a half days before the onset of spinning. The injection of phenylthiourea into silkworms during this pre-spinning period would be expected therefore further to depress the already decreasing rate of oxygen consumption. It is also reasonable to assume that this fall in the rate of respiration reflects the progress in the series of events of metamorphosis which is triggered off by the "pupation" hormone (Williams, 1947). The timing of the onset of spinning is therefore already under hormonal control, and the insertion of phenylthiourea into the blood just prior to the spinning period is unlikely to influence it.

Accordingly, the compound was injected in solution into *Cynthia* about 24 hours before spinning was due to begin. This stage was conveniently marked by

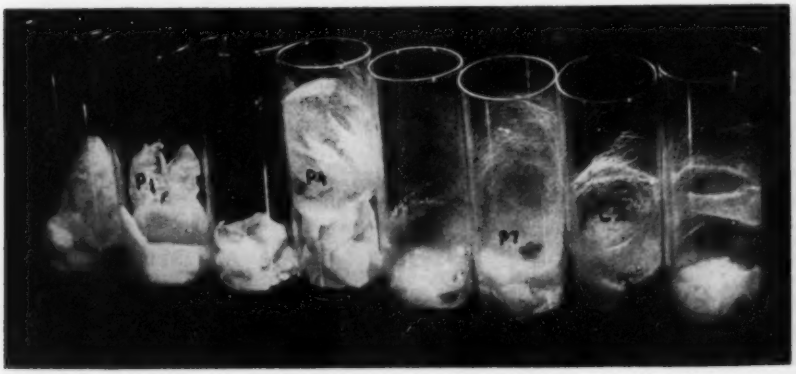

FIGURE 4. Series of structurally different cocoons spun by *Cynthia* after being injected 24 hours previously with varying amounts of phenylthiourea. From left to right in the photograph relative amounts of the inhibitor used were, nil, 0.1 mg., 0.1 mg., 0.3 mg., 0.4 mg., 0.5 mg., and 0.7 mg.

the tip of the spinneret turning black. The amounts of the compound introduced into the blood in these studies ranged from 0.1 to 0.7 mg.

The results in Table I illustrate a relationship between the depressant action of phenylthiourea on the rate of oxygen consumption at the beginning of spinning and the types of structures spun. It seems that the extent to which the rate of respiratory metabolism is decreased at the beginning of spinning determines the type of structure spun (Fig. 4).

Discussion

1. *Depressant action of phenylthiourea on respiration*

The results of this investigation show that the influence of phenylthiourea on the respiration of *Cynthia* is one of depression, which is proportional to the concentration.

During the course of normal metamorphosis in silkworms there is a striking fall in the rate of oxygen consumption. A considerable fall in the rate, however, also occurs at each moult, and it is significant that when a larva is deprived of food there is an accompanying decrease in the respiratory rate (Wilson, unpublished work). It seems, therefore, as if cessation of feeding by silkworms prior to metamorphosis may be partly responsible for the decrease in the metabolic rate.

The introduction of phenylthiourea into the blood of *Cynthia* just before the spinning phase markedly depressed the rate of oxygen consumption below the normal level. This obviates the possibility that the inhibitory effect of phenylthiourea on respiration is simply due to this compound halting feeding. The evidence instead converges in support of this compound exerting an inhibitory action upon some site in the respiratory chain of *Cynthia*. It seems unlikely, however, from previous work that the site of the depressant action is the cytochrome oxidase or succinic dehydrogenase systems (Grant and Krantz, 1942; DuBois and Erway, 1946).

Since the experimentally induced fall in the rate of oxygen consumption in *Cynthia* is accompanied by an inhibition of the blood phenolase activity, the possibility of this enzyme being implicated in some way with respiration, as suggested by Heller (1947), cannot be ruled out. Though potentially capable of serving as a terminal respiratory oxidase, it would, however, seem unnecessary for phenolase to perform this function in a silkworm in which there is an intact and functional cytochrome-cytochrome oxidase system. If one prefers to accept the possibility of phenolase serving as a terminal respiratory oxidase despite the presence of cytochrome oxidase, there then remains the further possibility that phenolase may be coupled with the oxygen which is supposed to diffuse from the tracheoles and across the short distances to the adjacent tissues.

It was suggested long ago, however, that the depressant action of urea derivatives on respiration is exerted on a part of the respiratory chain involving DPN, and not on the oxygen terminal part of the chain (Grant and Krantz, 1942). This is still believed to be the case (Slater, 1958).

Morton (1958) has recently drawn attention to the wide distribution of quinone compounds in tissues, and Slater (1958) has suggested how these compounds may be involved in oxidative phosphorylation. The evidence up till now is in favour

of this, and it is therefore tempting to suggest that if the quinones in question are derived from quinol compounds only in the presence of an active phenolase-type enzyme, then blocking this catalyst would be expected to have an inhibitory effect on oxidative phosphorylation. To speculate further than this is unlikely to be of much value because there are still many gaps in our knowledge about the processes involved in oxidative phosphorylation.

2. Relation of metabolic rate to spinning behaviour

It will be seen from the results shown in Table I that the differences between the cocoons spun by *Cynthia* after treatment with varying amounts of phenylthiourea are qualitative. This obviates the results being attributed to a general muscular debility. Moreover, during the spinning period the treated silkworms were as active as the controls. This recalls the observation of Van der Kloot and Williams (1954) that *Cecropia* retains its normal activity when exposed to carbon monoxide yet at the same time is rendered incapable of spinning a cocoon.

The results of the present work show that the type of cocoon spun depends on the extent that the rate of oxygen consumption is depressed at the beginning of the spinning phase by phenylthiourea. Since the cocoon is the end-result of a complicated pattern of neuromuscular activity, any deviation from normal in the structure of the cocoon may be taken as reflecting a change of behaviour. It is therefore reasonable to conclude that quantitative differences in the metabolic rate are coupled in the silkworm with differences in behaviour which are qualitative. It would be interesting to learn to what extent this principle can be extended to other forms of behaviour, whether instinctive or otherwise.

Summary

1. A symptom of the metamorphosis of *Cynthia* is that the precipitous fall in the rate of oxygen consumption begins two and a half days before the spinning period.

2. The rate of oxygen consumption is reduced to half the maximal rate of 1400 mm.^{3} O_2/gm. pupal wt./hr. when spinning begins. It continues to fall throughout the spinning phase and reaches a level of 75 mm.^{3} O_2/hr. in the early pupal stage.

3. Phenylthiourea has a pronounced depressant action on the rate of oxygen consumption, the depression being proportional to the amount of this compound introduced into the blood.

4. The decrease in respiration brought about by phenylthiourea coincides with the inhibition of blood phenolase activity. Possible sites of the depressant action of phenylthiourea on respiration are discussed.

5. When the rate of oxygen consumption is depressed to various levels at the beginning of spinning a series of qualitatively different cocoons is produced.

6. It is concluded that the pattern of spinning behaviour is delicately tuned to the metabolic rate.

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ON THE EMBRYONIC DEVELOPMENT OF THE SEA URCHIN ALLOCENTROTUS FRAGILIS [1]

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Allocentrotus fragilis, thanks to its brightly colored test and whitish short spines, has been termed the most beautiful of the sea urchins. Perhaps because of the fragility of the almost paper-thin test and the depths at which this sea urchin lives there was no study of the species before that of Jackson in 1912. He gave it the name *Strongylocentrotus fragilis*. In 1943 Mortensen, in his revision, assigned to the species a new generic title, *Allocentrotus* (Boolootian *et al.*, 1959).

The first specimens taken in any quantity at the Pacific Grove station were dredged from depths of 80 to 100 fathoms, temperature approximately 8° C. Although samples were taken throughout the year, I have found ripe eggs only in February and March. Animals dredged in mid-April showed that complete spawning had taken place, with the exception of one male which yielded a little active sperm. The animals lived very well for at least two weeks in aquaria of the laboratory in which the temperature of the running sea water was approximately 14° C. Eggs and sperm were taken from these animals from time to time. The specimens used were from 50 to 80 mm. in diameter. Only one of those taken during the three seasons equaled in diameter the largest recorded by Clark (1948), namely a little over 100 mm. The test of this specimen is in the collection of Dr. J. Wyatt Durham of the Department of Paleontology, University of California, Berkeley.

Eggs and sperm were first obtained by opening the animals and putting the gonads into dishes of sea water into which the sex products quickly escaped. Later, in order to avoid waste, the electrical method (Harvey, 1953) was used and found to be very satisfactory. The eggs are very light in color, so that, as they stream out of the ovary they may at first be mistaken for sperm. The diameter of the egg is approximately 110 μ which is midway between the dimensions of the eggs of the two shore forms, *Strongylocentrotus purpuratus* (78 μ) and *Strongylocentrotus franciscanus* (140 μ). The fertilization membrane closely invests the egg, being in distance from the egg's surface $\frac{1}{16}$ the diameter of the egg (Fig. 1). In contrast the fertilization membrane of *S. purpuratus* is removed from the surface of the egg $\frac{1}{6}$ the diameter of the egg.

At a given temperature, the eggs of *Allocentrotus fragilis* divide at exactly the same rate as those of the two shore forms *Strongylocentrotus purpuratus* and *S. franciscanus*. The temperature for successful development of *Allocentrotus fragilis* must be kept at 15° C. or lower (Moore, 1959) (Figs. 1–4). There is, however, some variation, the eggs of an occasional individual segmenting normally at 16°

[1] Identified for me by Dr. J. Wyatt Durham.

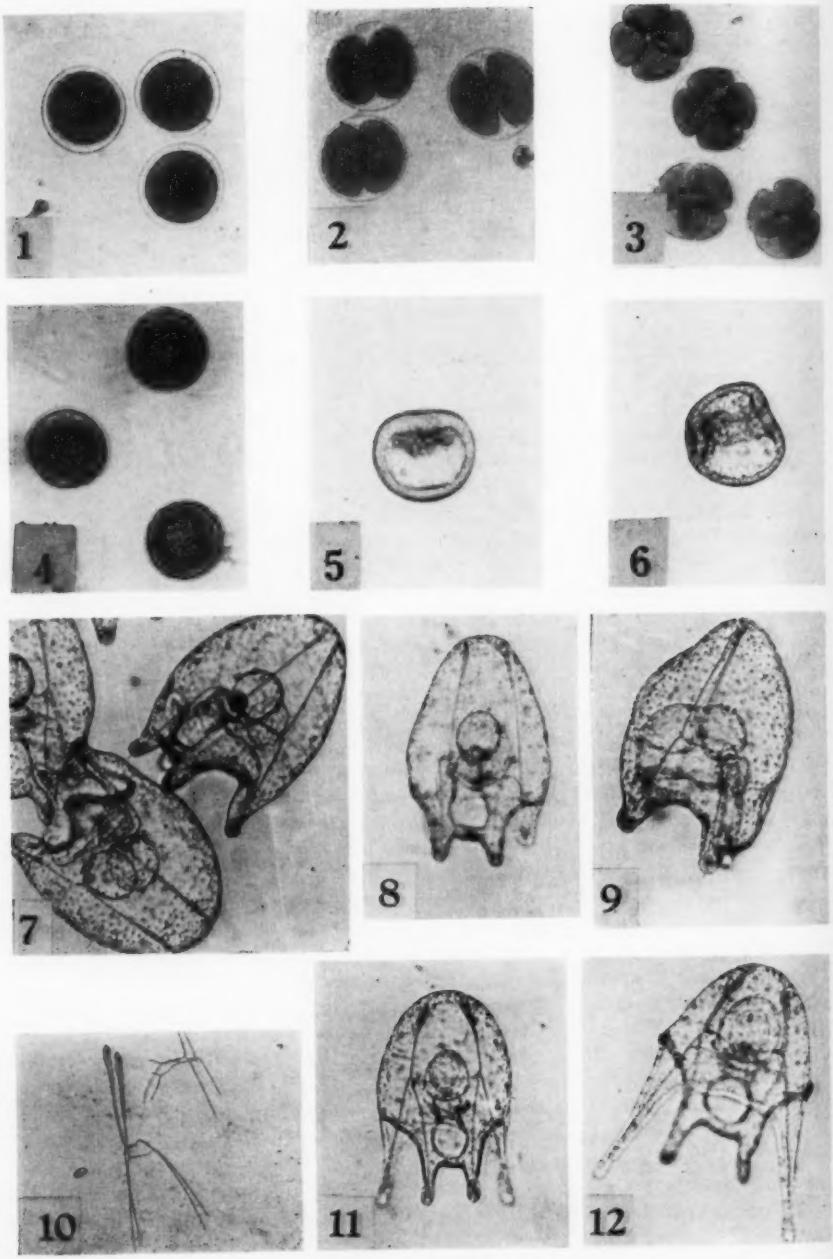
or even 17° C. while others require 14° C. or lower for successful development.

The blastulae (Fig. 5) early show flattening at the poles, in that way differing from the oval form of the same stage in *S. purpuratus*. The pluteus, which is formed three days after fertilization, resembles that of *S. purpuratus* in having short arms, but the shape of the body is somewhat cylindrical and thus differs from that of *S. purpuratus* which is conical or pyramidal in shape (Figs. 7, 8, 9). The skeleton (Fig. 10) resembles that of *S. purpuratus* in the club-shape of the body-rods, in its smoothness and in the short post-oral rods; the cross-bars are absent. In general the skeleton is thinner than that of *S. purpuratus*. An accessory post-oral rod is frequently present. In contrast to the comparatively simple structures of these two forms, the pluteus of *S. franciscanus* is more elaborate in that it is characterized by long arms, the presence of cross-bars and thorniness of the body-rods at the apex (Fig. 12).

Cross-fertilization with the two shore forms succeeded especially well in February. The eggs of *S. purpuratus* fertilized with the sperm of *A. fragilis* gave more than 50 per cent fertilization as did the reciprocal cross. The differences between the two forms in the characters of the pluteus are so slight that no study of these hybrids was made at this time. The case is different in the cross *A. fragilis* ♀ × *S. franciscanus* ♂. Here the percentage of the eggs fertilized varied between 20 and 50 per cent. Figure 11 illustrates a pluteus of this hybrid. The lengthening of the arms, the presence of cross-bars and thickening of the apical skeleton are the chief features derived from the male parent (Fig. 12). The hybrid therefore has longer arms and more complex skeleton than the maternal species by virtue of characters inherited from the male parent, and therefore follows the pattern of the cross *S. purpuratus* ♀ × *S. franciscanus* ♂ (Moore, 1943).

Discussion

The deep water sea urchin, *Allocentrotus fragilis* has been taken at depths of 40 to 417 fathoms from Vancouver Island to Pt. Santa Eugina in Lower California (Clark, 1948). The species thus can thrive at a very low temperature and in the absence of light. The short breeding season, February and March, raises the question of the triggering of spawning, which takes place promptly at the end of March or beginning of April. This is the record of three successive seasons at the Pacific Grove station, and leads one to believe that spawning is, for some reason, associated with the onset of spring. Increase of light or temperature as causative factors would seem to be excluded because of the conditions at the great depths at which this species flourishes. Pertinent to the question of the triggering of spawning is the fact that ripe individuals have been kept in laboratory aquaria at a temperature of approximately 14° C. for two weeks or longer without their showing any evidence of spawning. During the time the animals retained their spines, they yielded eggs and sperm. Thus, a sudden increase in temperature and abundant light did not show any effect of triggering the spawning of ripe individuals. As to the cause of spawning in the natural habitat, there is the possibility in the spring flowering of the plankton algae. Thorson (1946) has suggested that these algae may, by their fall to the bottom, yield algal extracts which furnish the chemical trigger for spawning. There remains the possibility



FIGURES 1–12.

that this animal, as a character of its being, has an annual periodicity which is independent of environmental factors.

The close relationship of *Allocentrotus fragilis* to the two shore forms of *Strongylocentrotus* is indicated, first, by the exact time relations of development, which are precisely the same in the three species, and by the fact that crosses can easily be made between *Allocentrotus fragilis* and the two species of *Strongylocentrotus*. The inheritance of paternal characters in the cross *Allocentrotus fragilis* ♀ × *S. franciscanus* ♂ proves that we have here true fertilization and not parthenogenesis.

It gives me pleasure to record my cordial thanks to Mr. Tom Fast who made the initial discovery of the bed of *Allocentrotus fragilis* at Pacific Grove in February, 1957, and who gave me the entire number taken at that time. Subsequent hauls were made by junior members of the Station staff who very graciously furnished me with specimens needed for my work.

Summary

1. Ripe eggs and sperm of *Allocentrotus fragilis* were obtained during February and March, from animals taken at depths of 80 to 100 fathoms.

2. Fertilization was approximately 100 per cent, and development was normal at a temperature of 15° C. or lower.

3. The rate of development in *Allocentrotus fragilis* is identical to that of the two species of *Strongylocentrotus* at this locality.

4. The pluteus has a characteristic form which distinguishes it from those of *S. purpuratus* and *S. franciscanus*.

5. Cross-fertilization between *Allocentrotus fragilis* and the two forms of *Strongylocentrotus* was accomplished, and in the progeny of *A. fragilis* ♀ × *S. franciscanus* ♂ the development of paternal characters was clearly shown.

6. As to the cause of spring spawning, increase in light and possible increase in temperature are not factors, but Thorson's idea of the occurrence of algal extracts of the plankton in early spring is suggested as a possible factor. There is also the possibility of an innate periodicity characteristic of the species.

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Figures 1 to 10 inclusive refer to *Allocentrotus fragilis*. Magnification 100 ×.

Figure 1. Fertilized egg.
Figures 2, 3. Segmentation stages, 2-cell and 4-cell.
Figure 4. Blastula before hatching.
Figure 5. Swimming blastula, 24 hours.
Figure 6. Gastrula, 48 hours.
Figures 7, 8, 9. Plutei, 5 days.
Figure 10. Skeleton of pluteus of *Allocentrotus fragilis*.
Figure 11. Pluteus of hybrid *Allocentrotus fragilis* ♀ × *Strongylocentrotus franciscanus* ♂, 5 days.
Figure 12. Pluteus of *Strongylocentrotus franciscanus*, 5 days.

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ACOUSTIC ORIENTATION IN THE CAVE SWIFTLET

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Acoustic orientation has been demonstrated in eleven families of bats of the suborder Microchiroptera (Griffin and Galambos, 1941; Galambos and Griffin, 1942; Griffin, 1958; Moehres, 1953; Moehres and Kulzer, 1955a, 1955b, 1957; Griffin and Novick, 1955; Novick, 1958a), in the Megachiropteran bat genus, *Rousettus* (Moehres and Kulzer, 1956; Novick, 1958b), and in the oilbird, *Steatornis caripensis* (Griffin, 1953). In each of these cases, the acoustically orienting individual produces and emits specialized clicks or pulses of sound which are reflected by intercepting surfaces such as obstacles or insect prey. The echoes from such surfaces are received by ear, interpreted, and the information used for navigation in pursuit of prey or avoiding obstacles. Griffin has called such an orientation system, based on the sounds which the navigating animal itself produces, echolocation. In the case of the fruit bat, *Rousettus,* and the oilbird, *Steatornis,* such acoustic orientation is facultative and is used to supplement vision in poor light. Among the Microchiroptera acoustic orientation is obligatory. In no known case, can these bats orient successfully by vision. While *Rousettus* and *Steatornis* probably need echolocate only obstacles and landmarks, some of the Microchiroptera have reached a high degree of specialization in tracking flying insect prey by echolocation. The occurrence of such a phenomenon among three such diverse groups suggested that acoustic orientation might prove to be common among vertebrates that fly in the dark. *Steatornis* and *Rousettus* are both nocturnal and cave-dwelling. Griffin (1953) found one chamber of a cave occupied by *Steatornis* to be totally dark at the time the birds were flying about successfully. Novick (1958b) found *Rousettus* living only in dimly lighted caves, but their ability to fly in total darkness has been shown experimentally (Griffin *et al.,* 1958). The Microchiroptera are all nocturnal and are frequently cave-dwelling as well.

Swiftlets of the genus *Collocalia* frequently inhabit caves, especially for nesting (Baker, 1934). Though their behavior in this respect is by no means uniform, many *Collocalia*-inhabited caves are known to be totally or almost totally dark. Though these birds feed diurnally and roost during the night, it seemed likely that, lacking the ability to echolocate, they would find cave flight difficult, especially in caring for their young. They would, moreover, be light-adapted each time they abruptly entered their cave, further reducing the usefulness of vision. Thus there appeared to be a strong likelihood that these birds would prove to be orienting acoustically under conditions of poor light.

In the course of an expedition which I undertook to study the orientation of the bats of the Old World tropics (Novick, 1958a, 1958b), I was able to observe the behavior of the cave swiftlet, *Collocalia brevirostris unicolor* in the vicinity of Namunukula, Uva Province, Ceylon in April, 1956. In addition, I observed swiftlets, *Collocalia* sp., in Cavite and Bataan Provinces in the Philippines between

November, 1955 and February, 1956. Griffin (1958) has reviewed the preliminary reports from this expedition.

This work was supported in part by the Office of Naval Research, the United States Public Health Service, the Sigma Xi-RESA Fund, the Belgian American Educational Foundation, and Harvard University. During this period, I was a Fellow of the National Institute of Neurological Diseases and Blindness and a Research Fellow in Biology at Harvard University. I gratefully acknowledge the aid given me by the personnel of the United States Navy and Air Force in the Philippines and by the personnel of the American Embassies in the Philippines and Ceylon. I am most deeply indebted to Major W. W. A. Phillips of Namunukula, Ceylon, for his help in observing, capturing, identifying, and studying the swiftlets, and for the kind hospitality which he and Mrs. Phillips extended to me. His many years of devoted study of the birds (and mammals) of Ceylon proved invaluable. Dr. D. R. Griffin of Harvard University not only drew my attention to the question of orientation in *Collocalia* but gave me advice and support of every description.

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Methods

The swiftlets were captured on several occasions in the Philippines and Ceylon in fine-mesh mist nets draped over cave entrances. Birds were caught both entering and leaving the caves. In Ceylon, *Collocalia* were recorded in their natural environment and in the laboratory, using an Amplifier Corporation of America Magnemite, model 610-E, portable tape recorder and crystal microphone. While the results fall short of ideal, more satisfactory equipment was not available. Two birds were also recorded in captivity, using a battery-operated 640 AA Western Electric condenser microphone with cathode follower, a custom built amplifier, two Krohn-Hite variable band-pass filters, models 310-A and 310-AB (total slope 48 db/octave), and a Dumont oscillograph, model 304H. The oscillograph screen was photographed with a Bell and Howell motion picture camera, model 70-DA. The birds were recorded in flight in partial light and in the dark. One was also recorded escaping from a net placed near the microphone.

In determining the birds' dependence on vision and hearing, cotton pellets fixed in place with collodion were used for blindfolding and ear plugging. These proved satisfactory and relatively easy to apply and remove without significantly injuring the bird.

The birds in Ceylon were identified in life by W. W. A. Phillips. In deference to the prevailing Buddhist religion, none were killed. The literature on the natural history and taxonomy of *Collocalia* is scattered but some interesting comments can be found in Baker (1934), Manuel (1937), Mayr (1937), Lack (1956), and Medway (1959).

Observations

The bulk of the observations herein reported concern a colony of *Collocalia brevirostris unicolor* nesting in Hindigalle Cave, Hindigalle Group, Namunukula,

Ceylon. This cave is just below the summit of a hill and has a main entrance that is about four feet high and perhaps ten feet across, as well as several smaller openings along the same side of a roughly rectangular chamber. The walls and ceiling are irregular rock with few niches or crevices. There is a dirt floor. The cave, on this occasion, was damp but had no running water. It consists of a single chamber about forty feet deep, twenty-five feet wide and from five to ten feet high, and contained, at that time, several dozen nests on the ceiling at its inner, most dimly lighted end. Some of these nests looked old and neglected and may have been the remnant of the previous season. On April 5, 1956, the majority were occupied by one or two eggs or by one or usually two young, ranging in age from newly hatched to almost adult size. The nests were of uniform architecture having a large foot attached to the ceiling with an underslung bowl-shaped shelf. The basic salivary structure was reinforced with bits of moss-like material.

The light in the cave was sufficient for me to make out gross detail even while I was light-adapted. From a position near the nests, the light measured 100 units on a Weston light meter facing the entrance and 0.1 unit facing in toward the darkest corner. Standing immediately inside the principal entrance and facing inward, the light registered at 0.8 unit. This, of course, would vary with the time of day and the weather. The Weston light meter measures in candles/sq. ft. Unfortunately this is a reflected light meter.

The swifts proved reluctant to enter while I was inspecting the cave but entered readily after I had secreted myself in a corner. The birds could be seen flying about outside in pursuit of insects, and could be heard making an occasional high pitched call. As one or more flew through the cave entrance, however, they abruptly began to emit relatively low pitched clicks at a rate of a few per second. These clicks seemed uniform in quality but varied in repetition rate as the birds flew about. The rate increased as the birds reached the darker end of the cave and reached its highest point as they circled the nests and landed. In each case, when a bird turned toward the entrance to leave the cave it ceased clicking though it still had the full length of the cave to traverse.

I was able to clip a small microphone to one of the occupied nests in the center of the group of nests and, using a long cord, withdraw to a corner with the tape recorder. Though the birds were clearly disturbed by my presence and by the noise of the running recorder, they entered the cave and flew about often enough for me to record their vocalizations.

At a second location near Namunukula, Ceylon, I was able to observe nesting swiftlets enter a large, high cleft in a cliff face to tend their nests. I could not approach the nests in this case and recorded the birds as they flew through the cleft at a distance of 50 or 60 feet from me and the microphone. This cleft was nowhere dark but was quite dimly lighted to my light-adapted eyes.

Three swiftlets were captured at Hindigalle Cave and carried to a temporary laboratory which was a room about 25 feet long, 12 feet wide and about 8 feet high at the walls, with a peaked and beamed ceiling, rising some four or five feet to its apex. The walls were light colored; one wall consisted of windows. The beamed ceiling was of very dark wood. When the birds were released in this room in good light they flew silently unless they rose above the level of the painted wall into the darker ceiling peak. There they produced clicks resembling those

heard in the cave. When the blinds were drawn over the windows, the birds clicked more often. In the dark or very dim light, at night, they clicked continually as long as they flew about but not when roosting. They clearly preferred not to fly in poor light and usually roosted quickly, taking flight again only when forced to do so. When the eyes of one bird were thoroughly blindfolded, the bird flew slowly about the room with an appearance of caution, following closely the contours of the walls and ceiling and clicking continually. The ears of one bird were plugged and, in the dark, this bird flew in a bewildered manner and roosted whenever possible. It appeared not to crash into obstacles as bats under similar conditions do but to flutter in a cautious manner until it came up against an obstacle and then catch hold. This bird was very reluctant to fly when deaf.

The sounds produced by these birds resemble those made by a child's rapidly rotated ratchet toy. There is a principal frequency of about 4–5 kc. accompanied by many overtones. Because of the differential sensitivity of the microphones used, these records cannot be analyzed too rigorously for the complete frequency spectrum. The recorded sounds were initiated by a high amplitude portion of relatively pure frequency of 2–6 msec. duration, followed by a low amplitude portion of highly complex wave form lasting for up to 30 msec. or more. The high amplitude portion was almost surely a direct record of the bird-emitted sound but its apparent duration and amplitude depended upon the bird's orientation relative to the microphone and its distance. The low amplitude portion may well have included echoes from adjacent surfaces. The repetition rate observed varied considerably too. In no case was a sequence of more than 2–3 seconds recorded at a favorable signal:noise ratio. In one 2-second sequence in the cave, 15 pulses occurred—a rate of 7.5/sec. In another long sequence, the rate was 6/sec. In others the rate varied from almost 10 to about 5.5/sec. Within these series, the rhythm was not regular but the pulses were apparently spaced from 60 to 300 msec. apart. The long intervals may well have represented a missing click (when the bird turned away from the microphone). These records were made of birds circling their nests when, by ear, their repetition rate was most rapid. Further studies of these birds are required to measure frequency, duration, repetition rate, and rhythm more accurately.

In the Philippines, I had occasion to observe a number of colonies of *Collocalia* sp. Only one specimen was collected and preserved. R. A. Paynter of the Museum of Comparative Zoology of Harvard University was reluctant to make a positive identification of a single specimen of this difficult group. In any case, these colonies were observed at Cabag Cave, Luksuhin, Silang, Cavite Province; a coastal cave south of Ternate, Cavite Province; and a great number of small tidal caves and overhangs along the coast west of Mariveles, Bataan Province, Luzon. These swiftlets are very abundant in central Luzon. In every case, the cave which they occupied was dimly lighted or well shaded, never fully lighted nor totally dark. In some cases, the nests were on the ceiling; in others the nests were on the walls at about 6 to 8 feet above the floor, but in all cases, the nests were so placed that they were shaded from the entrance of the cave and in the darkest locations. I was able to observe closely a colony in an abandoned, man-made tunnel near Mariveles, Bataan. The tunnel was well lighted throughout, during the day, by sunlight but at the rear, behind large steel ceiling beams, I found

several clusters of nests, some of which contained one or two eggs or young of various stages of development. The nesting corners were dim but not dark. I could examine the nests without artificial light. These birds never produced any audible vocal clicks in the tunnel or in any of the other caves I visited. Nor could I detect any inaudible utterances with a custom built pulse detector and Rochelle salt crystal microphone (Griffin and Novick, 1955) sensitive to frequencies between 10 and 100 kc. On one occasion, a single bird, somewhat differently marked from the rest, clicked like *C. brevirostris unicolor*. I was unable to capture or relocate this individual. At night the terrain outside this tunnel was floodlighted to prevent the theft of quarry equipment. The tunnel was dark but not totally so. If the birds were disturbed (and a bright light was the most effective way of doing so) they flew out and circled the outside lights for long periods before returning to their roosts one by one. No clicks were observed. Next to the occupied tunnel on one side was a similar but better lighted tunnel which had only a few swiftlet nests in dim corners. On the other side was an almost totally dark tunnel that could be entered easily but had no swiftlets in residence or any evidence of swiftlet occupation in the past.

Discussion

The occurrence of acoustic orientation and echolocation in *Collocalia brevirostris unicolor* is thus established to a reasonable certainty. These birds emit clicks of a design resembling those of *Steatornis* and *Rousettus*. These clicks are emitted only in poor light and the repetition rate increases as the light decreases. This relationship was observed in the cave, where the repetition rate increased as the bird flew deeper into the cave, and in the laboratory, where the rate increased not only with decreased direct light but as the background became dark (the dark-painted peaked ceiling). Thus there appears to be a reciprocal relationship between dependence on vision and on hearing for orientation. Flight in the dark or blindfolded is cautious but competent. Flight when the ears are plugged is incompetent in the dark.

It would appear, further, that echolocation is reserved by *Collocalia* for flight in caves. These birds are all diurnal and give no evidence of producing an echolocating type of sound while in flight in the daylight. In nature, it is only when they enter their caves that the typical sounds are emitted. Obviously, cave dwelling imposes special needs on a flying vertebrate. These birds must emerge to find sufficient food. Their diurnal hunting and flight are well facilitated by vision. When they re-enter their caves, however, they are not only light-adapted but, of course, are in dim light or even total darkness (Baker, 1934; Rabor, personal communication, quoted in Griffin, 1958). Thus, to find their nests without accident, they must supplement vision with an orientation system independent of light. Just such a system has been evolved as well by three other groups, *Steatornis, Rousettus,* and the Microchiroptera, all of which fly in darkness. A satisfactory orientation system for flight in darkness must be independent not only of light but of touch for, of course, the animal is out of contact with its physical surroundings except for the air. The information-carrying energy must move rapidly relative to the animal's movement, for otherwise timely and precise information would be lacking. Smell is unlikely to suffice though it may supplement echolocation to

the extent of identifying the goal (nest, roost, edible fruit, etc.) when the animal has reached its immediate vicinity; otherwise odors not only move too erratically (depending upon air currents) but are not, of course, given off by all dangerous obstacles. Dependence on random sounds alone for informative echoes would be haphazard and insufficiently precise, just as urban men cannot depend on random light at night for finding their way on streets or in automobiles. So the system which appears to fit the logical needs is, indeed, the system which has evolved repeatedly—the dependence upon the echoes of self-produced sounds to inform the flying animals in the dark about their surroundings.

Not all species of *Collocalia* enter deep caves. Many nest routinely in shallow caves or under overhangs. I should speculate that not all species of the genus are capable of acoustic orientation and that this lack restricts their nesting sites to those that are adequately lighted. Further observations on *Collocalia* nesting and orientation are clearly necessary.

In addition, further studies of the accuracy of *Collocalia* echolocation, the mechanism by which these sounds are produced, and the versatility of the system are indicated.

Conclusions

1. The cave swiftlet, *Collocalia brevirostris unicolor,* orients acoustically in poor light or when blindfolded but orients visually in good light.

2. In nature, these swiftlets orient acoustically when they enter and fly about their dimly lighted or dark caves.

3. They emit sounds of characteristic pattern of about 4–5 kc., with an initial high amplitude portion of about 2–6 msec. duration followed by a long, low amplitude portion of undetermined significance. The maximum repetition rate appears to be about 5–10 clicks/sec. The repetition rate varies inversely with the amount of light and increases when the bird encounters obstacles.

4. The echolocation system of the cave swiftlet resembles very closely that of the oilbird, *Steatornis,* and the fruit bat, *Rousettus.*

5. Echolocation appears to be common among vertebrates that fly in darkness. Echolocation in *Collocalia, Steatornis,* and *Rousettus,* however, differs from that in the Microchiroptera not only in the design of the outgoing pulses but by coexisting with accurate, functional vision.

6. In view of the failure to elicit evidence of echolocation in a Philippine species of *Collocalia* and the habit of many species of *Collocalia* of nesting in shaded but well lighted sites, echolocation may have evolved within the genus *Collocalia.*

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ON THE PRESENCE OF MYOGLOBIN AND CYTOCHROME OXIDASE IN THE CARTILAGINOUS ODONTOPHORE OF THE MARINE SNAIL, BUSYCON[1]

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Early studies of cartilage metabolism revealed the tissue to be almost completely devoid of aerobic oxidative metabolism (Kuwabara, 1932; Bywaters, 1937). That aerobic processes do occur, however, was subsequently shown by Boyd and Neuman (1954), who found chondroitin sulfate synthesis to be accompanied by significant oxygen utilization in chick embryo cartilage, and by Person and Fine (1959a, 1959b), who more recently demonstrated cytochrome oxidase and succinoxidase activity in invertebrate and vertebrate cartilages from very young animals. Additional evidence for the existence of aerobic processes in an invertebrate cartilaginous tissue will be presented in this report.

It was observed initially (Lash, 1959) that the odontophore, a cartilaginous structure which supports the radula of the whelk, *Busycon canaliculatum*, was colored red. On analysis, the red color was found to result from the presence of myoglobin in the tissue. The myoglobin was very similar to that found by Ball and Meyerhof (1940) in the radular musculature of the same animal. Additional studies revealed that the tissue possessed readily demonstrable cytochrome oxidase activity. In this paper we present a spectrophotometric characterization of the cartilage myoglobin and its pyridine hemochrome, and evidence for the existence of cytochrome oxidase activity in homogenates of the tissue.

Materials and Methods

Odontophores were obtained by knocking off the hard calcareous shell of the animal and exposing the proboscis. The proboscises were cut off, the odontophores dissected out, carefully trimmed of adherent muscle, and placed on aluminum foil in a beaker of cracked ice. Complete removal of muscle tissue from the odontophore was checked by means of a magnifying lens and also by examination of histological sections. For spectrophotometric and manometric studies the tissue was homogenized in a ground-glass homogenizer in 0.1 *M* $Na_2HPO_4-KH_2PO_4$ buffer, pH 7.4, or water, and used immediately. Some preparations were lyophilized immediately following homogenization in glass-distilled water.

Spectrophotometric studies were made with a Beckman Model DU Spectro-

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photometer and a Process and Instruments Recording Spectrophotometer, Model RS 3. For determinations of cytochrome oxidase activity, the oxidation of reduced cytochrome *c* by the tissue homogenate was followed at 550 mμ (Wainio *et al.*, 1951). Manometric determinations of oxygen uptake in a system limiting for cytochrome oxidase were made using hydroquinone as substrate in the presence of added cytochrome *c* (Sigma) (Eichel *et al.*, 1950).

Experimental and Results

1. *Absorption spectra of myoglobin*

The odontophore myoglobin was very soluble and easily dissolved out of the tissue by either glass-re-distilled water, or 0.1 *M* PO_4 buffer at pH 7.4. The curves presented in Figure 1 were obtained by homogenizing 236 mg. wet weight of freshly dissected tissue in 5.0 ml. of buffer at 4° C. The homogenate was spun in the centrifuge at 4° C. at 1700 × g for twenty minutes. The supernatant solution was decanted and used for spectrophotometric study. Reduction of the pigment was accomplished by the addition of sodium dithionite.

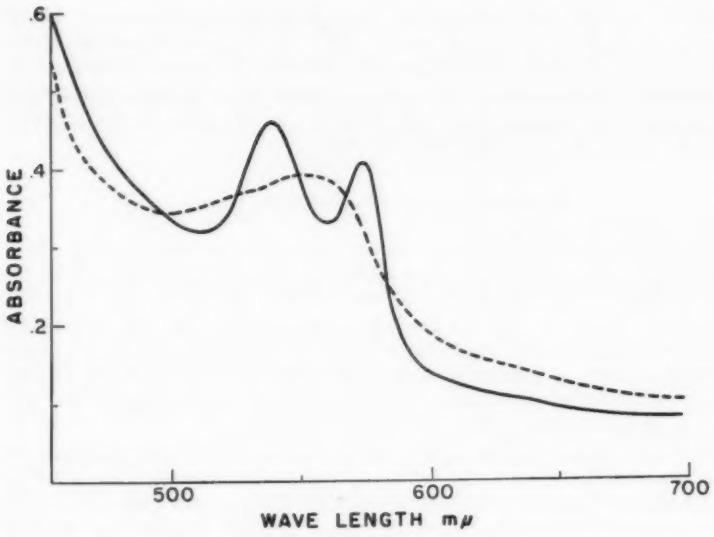
In Figure 1 are shown tracings made from original records of oxidized and reduced visible spectra, obtained in the recording spectrophotometer. The oxidized pigment (solid curve) exhibits an α peak at 574–575 mμ and a β peak at 538–539 mμ. Following dithionite reduction (dashed curve), a single broader and flattened absorption occurs at 540–565 mμ. These absorption maxima are similar to those determined (with a hand-spectroscope) by Ball and Meyerhof (1940) for the muscle myoglobin of Busycon, *i.e.*, oxidized compound, α peak, 570–580 mμ; β peak, 540–545 mμ. The discrepancies in location of the peaks may be the result of difference in instrumentation.

For study of the ultraviolet absorption of the myoglobin, the solution described above was diluted 1 : 5 with the same phosphate buffer. In Figure 2 the ultraviolet spectrum is shown. In the oxidized form (solid curve) a γ or Soret peak is present at 415–416 mμ. A broad, flat elevation encountered at 320–360 mμ is associated with the porphyrin moiety of the pigment (Barron and Flood, 1952). The sharper peak at 290 mμ is due to the presence of protein. A well-defined peak is seen at 238–239 mμ, possibly due to the presence of fatty acids in the preparation.

The spectrum of dithionite-reduced material exhibits a γ peak at 430 mμ; the remainder of the ultraviolet spectrum could not be obtained in the dithionite-reduced material because of the absorption of ultraviolet light by dithionite itself.

2. *Absorption spectra of pyridine hemochrome*

For preparation of the pyridine hemochrome of the myoglobin prosthetic group, 230 mg. wet weight of freshly trimmed odontophore were homogenized at 4° C. in 5 ml. of reagent pyridine (Merck and Co.). The homogenate was spun at 4° C. at 1700 × g for twenty minutes, and the supernatant solution decanted into a cuvette. Reagent pyridine was used in the reference cuvette. The visible absorption spectrum of the reduced hemochrome is shown in Figure 3. The α peak is located at 554–555 mμ, the β peak at 524–525 mμ. A broader and lower peak is present at 480 mμ. For determination of the ultraviolet absorption, shown in Figure 4, the pyridine supernatant described above was diluted 1 : 12 with addi-



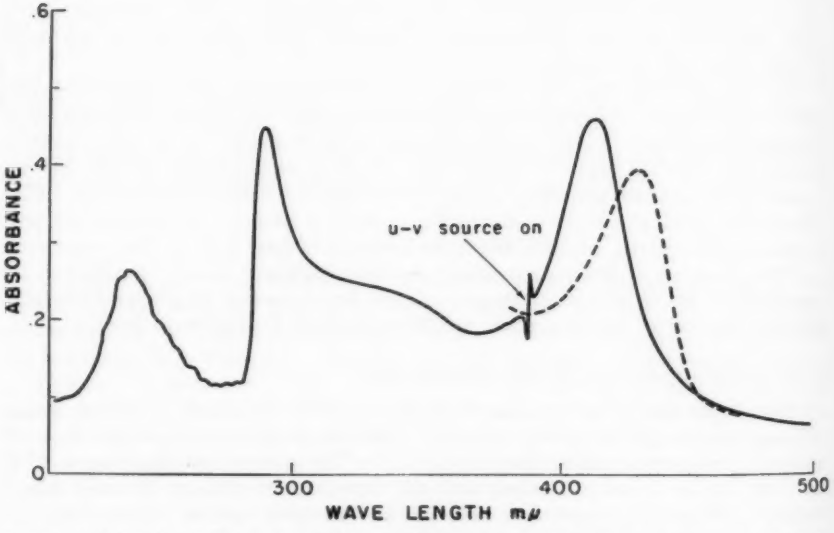
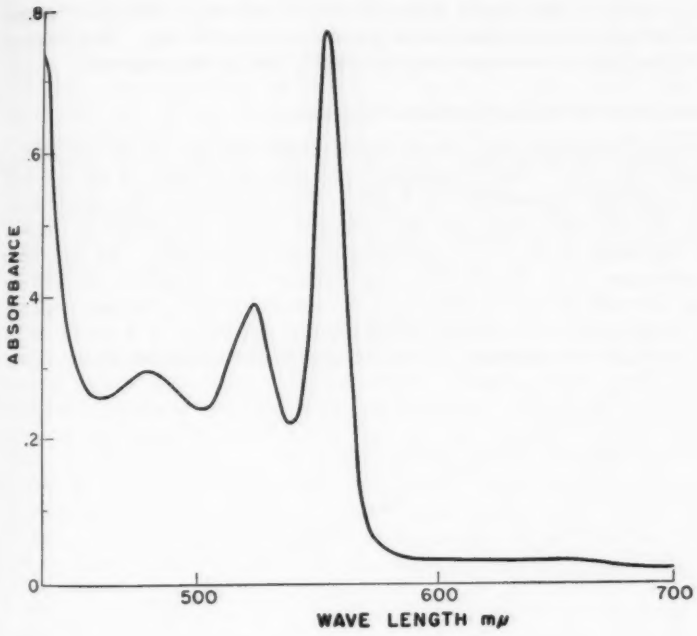


FIGURE 1 (above). Visible absorption of odontophore myoglobin. Solid curve: oxidized pigment; dash curve: dithionite-reduced pigment.

FIGURE 2 (below). Soret and ultraviolet absorptions of odontophore myoglobin. Solid curve: oxidized pigment; dash curve: dithionite-reduced pigment.



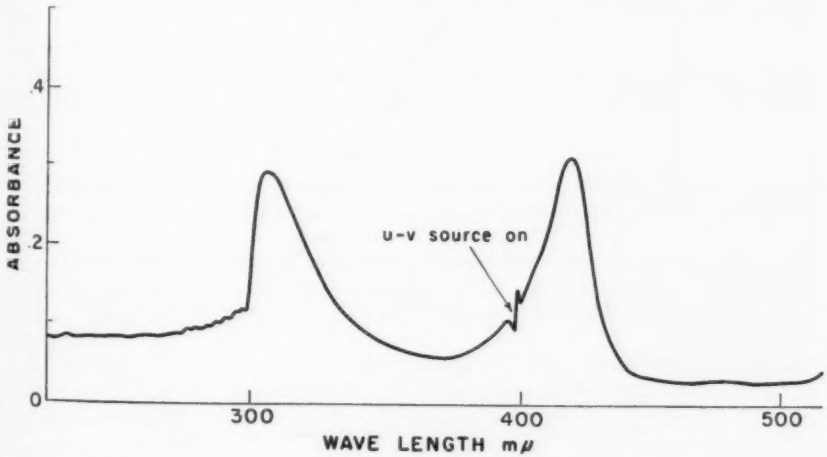


FIGURE 3 (above). Visible absorption spectrum of pyridine hemochrome prepared from odontophore myoglobin.

FIGURE 4 (below). Soret and ultraviolet absorptions of pyridine hemochrome prepared from odontophore myoglobin.

tional reagent pyridine. The γ peak is located at 417–418 mμ. The peak located at 290 mμ in the aqueous extracts is now present at 306–307 mμ. The peak at 238–239 mμ in the aqueous extracts is not present in the pyridine extracts.

3. *Cytochrome oxidase activity of cartilage homogenates*

In the spectrophotometric assay, tissue was homogenized in 0.1 *M* KH_2PO_4–Na_2HPO_4 buffer, pH 7.38. The homogenate was spun at 25,000 × g for one-half hour and the sediment re-suspended in buffer. The suspension was again spun at 25,000 × g for one-half hour and the sediment again suspended in buffer. Temperature was kept at 2°–4° C. throughout these operations. In this way endogenous substrates were removed and also most of the myoglobin, so that its absorption was not sufficient to interfere with the assay. Figure 5, curve 1, shows the decrease in absorbance of reduced cytochrome *c*, produced as a result of its oxidation by the tissue homogenate. Curve 2 depicts the inhibition of the above

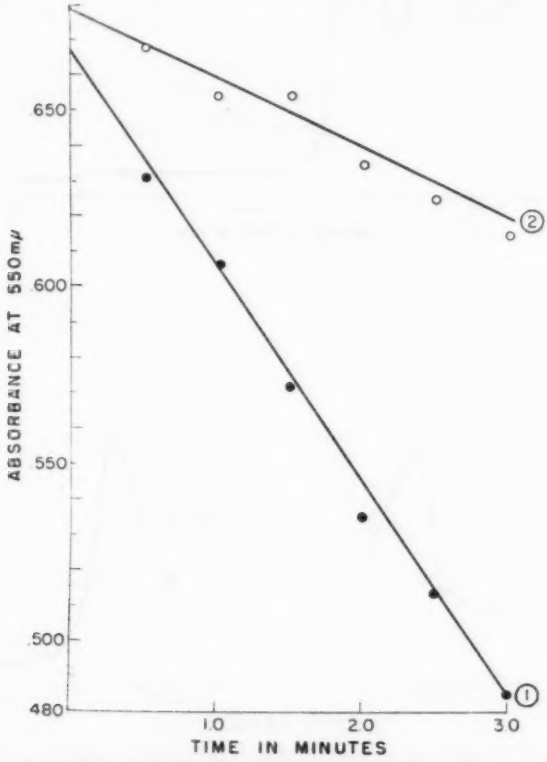


FIGURE 5. Cytochrome oxidase activity of odontophore homogenates as determined (spectrophotometrically) by oxidation of reduced cytochrome *c*. Curve (1): untreated homogenate; Curve (2): homogenate in presence of 10^{-4} *M* azide.

system by the incorporation of 10^{-4} *M* azide (final concentration). Whereas in three minutes a decrease of 0.171 absorbance units occurred in the absence of inhibitor, in the presence of 10^{-4} *M* azide, a decrease of only 0.058 absorbance units took place, or an inhibition of 66%. Similarly, 10^{-4} *M* cyanide (final concentration) produced a 75% inhibition of the cartilage oxidase activity. The cytochrome oxidase activity of homogenates prepared in the above manner was extremely labile. When kept at 0°–1° C. for 45 minutes, 50% loss in activity was noted. Homogenates in a thin-walled test tube, which were immersed in a boiling water bath for 25 minutes, were completely inactive. In a standard manometric assay using hydroquinone as substrate and added cytochrome *c*, the Q_{O_2}/(mg. dry wt.) of homogenates was 9.4 at 37° C. in an air atmosphere.

Discussion

The combined presence of myoglobin and cytochrome oxidase in a cartilaginous tissue is indeed interesting. The occurrence of hemocyanin as the blood oxygen-transport pigment in the same organism makes an unusual combination of oxygen transport and respiratory pigments, as was noted earlier by Ball and Meyerhof (1940). Since the physico-chemical properties of this myoglobin have not yet been studied, its exact physiologic role is not known. However, function as an oxygen-carrier and storage pigment for the cytochrome oxidase in the tissue appears likely. We have observed that the odontophores of younger animals contain less myoglobin than do those of older animals. As the snails increase in age and size, the odontophores take on a deeper pink to red color.

The presence of cytochrome oxidase in the tissue is indicated by its ability to oxidize reduced cytochrome *c*. We have been able to detect such activity in other invertebrate and vertebrate cartilage tissues as well (Person and Fine, 1959a, 1959b). Attempts to identify the characteristic absorption spectra of the oxidase have been unsuccessful thus far.

Other studies of the polysaccharide components of the odontophore (Lash, 1959) have shown that chondroitin sulfate could not be demonstrated in the tissue.

Summary

1. Myoglobin and cytochrome oxidase activities were shown to exist together in a cartilaginous tissue for the first time, in the odontophore of *Busycon canaliculatum*.

2. The absorption spectra of the cartilage myoglobin were characteristic for this class of pigments. Similarly, the absorption spectra of the pyridine hemochrome prepared from the pigment were characteristic of ferroprotoporphyrin-pyridine hemochrome.

3. The Q_{O_2} (dry weight basis) of cartilage homogenates, employing hydroquinone as substrate and added cytochrome *c*, was 9.4 in an air atmosphere at 37° C. Such homogenates were also capable of oxidizing reduced cytochrome *c*.

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ELECTRON MICROSCOPE STUDY OF THE DISTAL PORTION OF A PLANARIAN RETINULAR CELL[1]

NEWTOL PRESS

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With the increasing development of techniques of electron microscopy, ever more attention has been devoted to the study of the fine structure of photoreceptors, the details of which are beyond the limit of resolution of the light microscope. One such structure is the visual receptor of the eye of the planarian, *Dugesia tigrina*.

The classical study which has served as a model for many textbook illustrations of the planarian eye is the work of Hesse (1897), whose description of the visual end organ was subsequently modified by Taliaferro (1920). The latter investigator also posed the possibility of analogizing, and possibly homologizing, the retinulae of the turbellarian eye with the receptors of the vertebrate eye. Analogies between vertebrate and invertebrate eyes have been extended by Wolken (1958), who deemed the fine structure of the sensory cells of the planarian eye to resemble the outermost portions of vertebrate retinal components. Since preliminary studies by the present author tended to contradict this view, an electron microscopic examination of the planarian eye was deemed useful not only from the point of view of clarifying its morphology, but also from the standpoint of providing evidence as to a possible analogy of platyhelminth and chordate eyes.

Materials and Methods

Specimens of *Dugesia tigrina* were cut in two transversely at a level just behind the auricles, and the anterior portions were fixed immediately in a solution of 1% osmium tetroxide buffered at a pH of approximately 7.2 with a veronal acetate buffer. Following a period of fixation ranging from 20 minutes to 2 hours, the specimens were washed in distilled water, dehydrated in ethanol, and embedded in a mixture of 30% methyl methacrylate and 70% N-butyl methacrylate.

Sections, cut on an International Minot rotary microtome set to cut at 0.025 μ, were mounted on grids previously coated with a thin collodion membrane. The electron microscope used was an RCA model EMU 2.

Material for study with the light microscope was fixed in Bouin's fixative, dehydrated in ethanol, and embedded in paraffin. Sections were cut at 6 μ and stained with Heidenhain's iron hematoxylin.

Description

Observations with the light microscope show that the portion of the retinula (R, Fig. 1) found within the pigment cup exhibits a different capacity for staining

[1] This study was supported in part by a grant from the Research Committee of the University of Wisconsin with funds from the Wisconsin Alumni Research Foundation. I am indebted to Professor Paul Kaesberg of the Department of Biochemistry, University of Wisconsin, for allowing me to use his electron microscope facilities.

FIGURE 1. Light micrograph of an approximately frontal section, showing the pigment cup (PC), covering of the aperture of the pigment cup (C), retinula (R), nucleus of retinula (RN), and process of retinula (RP). The space between the most distal portions of the retinulae and the inner surface of the pigment cup is probably an artifact induced by fixation. × 1870.

FIGURE 2. Electron micrograph of an approximately longitudinal section through a retinula, showing the retinular process (RP), the lamellae (L) of the "middle region" (M), and the bulb-like swellings (RS) at the distal portions of the lamellae (RL) of the rhabdome (RH). A delicate membrane (MS) separates the middle and rhabdome regions of the retinula. × 20,900.

FIGURE 3. Electron micrograph of an approximately longitudinal section through a retinula, showing the lamellae (L) of the "middle region" (M), and the lamellae (RL) of the rhabdome (RH). Portions of rhabdomes of other retinulae are evident in the upper corners of the figure. × 19,000.

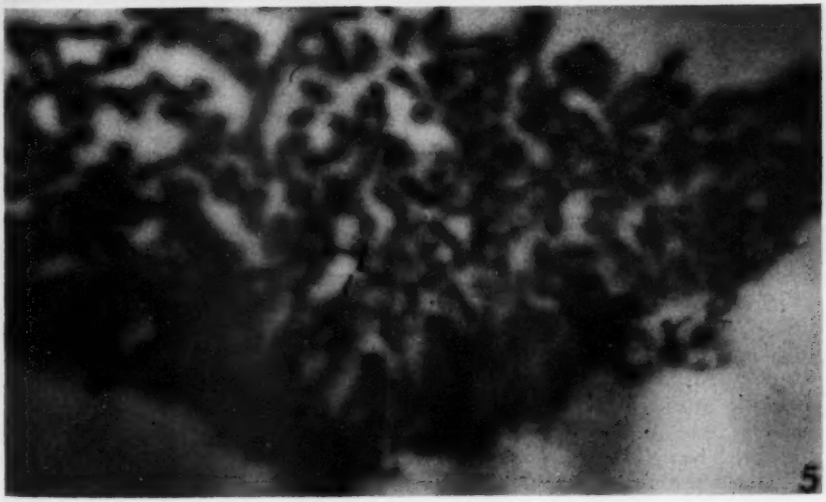

FIGURE 4. Distal portion of rhabdome of retinula seen in Figure 2, here shown at higher magnification, indicating the lamellae (D) in the distal swellings of rhabdome lamellae (RL). × 35,000.

FIGURE 5. Approximately transverse section through a portion of the "middle region" of a retinula, showing the lamellae (L) of that region. × 30,000.

with Heidenhain's iron hematoxylin from the rest of the retinula. The most distal portion of the sensory cell, which has been called the rhabdome by Taliaferro (1920), retains little of the stain and exhibits no internal structure save what has been termed a "rod border" (Hesse, 1897) or longitudinally oriented "striae" (Taliaferro, 1920). Proximal to the rhabdome of the retinula is a "middle region" staining intensely with iron hematoxylin, which continues proximally as a narrow process of the retinula. This latter process remains moderately intensely stained.

The electron microscope studies reveal that the whole of the rhabdome of the visual sensory cell consists of longitudinally oriented lamellae (RL, Figs. 2–4). These lamellae range in length from 3.18 μ to 4.20 μ, and in thickness from 300 Å to 490 Å. The distal portions of some lamellae are further differentiated into bulb-like swellings (RS, Fig. 2) bearing smaller lamellar structures with a maximum length of 0.44 μ that vary in thickness from 140 Å to 230 Å (D, Fig. 4).

A delicate membrane appears to delimit the "middle" from the rhabdome of the retinula (MS, Fig. 2). The sole discrete structures within the "middle region" (M, Figs. 2 and 3) are fiber-like lamellae (L, Figs. 2, 3, and 5). These lamellae vary in thickness from 160 Å to 610 Å, and appear in several instances to extend into the rhabdome.

Discussion

It has been suggested that the rhabdome of the retinula is its photosensitive region (Hesse, 1897; Taliaferro, 1920). In the visual biochemical reactions which presumably occur in the rhabdome, a lamellar arrangement would present a large surface area, particularly in a direction perpendicular to the longitudinal axes of the rhabdome vesicles. However, Taliaferro, in his experiments involving the locomotor responses of *Planaria* to light, has concluded (p. 113) that, "Light must strike a given rhabdome parallel with its longitudinal axis in order to cause stimulation of the rhabdome." But this direction is precisely the one which would least directly strike the longitudinal lamellar surfaces of the rhabdome. On the other hand, it is the most favorable direction by which light may reach the bulb-like swellings at the distal portions of the rhabdomal lamellae. The differentiated areas at the distal portions of the rhabdomal lamellae may be significant as possible sites of visual biochemical reaction.

The existence of a "middle region" as a distinct structure in the retinula of the planarian eye was not recognized by Hesse (1897), who figured in its place a fibrillar structure which was continuous distally with the "rod border" and proximally with fibers running to the cell body. In the preceding year Jänichen's (1896) description of the planarian eye showed a middle region in the retinula. In 1920, Taliaferro emphasized the importance of the "middle region," stating (p. 105), "that possibly this region serves as a crude lens to concentrate the rays of light upon the sensitive rhabdome and that photic stimulation depends upon this." Another possibility, however, is that these lamellae may transmit the impulse propagated by light-stimulation of the rhabdomal constituents. The latter explanation finds some support in the fact that some lamellae can be seen to continue into the rhabdome region.

As for the possibility of analogizing or homologizing the turbellarian and chordate eyes, the most that can be said is that certain similarities do exist be-

tween the two types. Both kinds of eye are of the inverse type, having the most distal portions of the receptors directed away from the opening of an eye partially lined with opaque pigment. Both kinds have visual receptors, each consisting of at least three distinct portions. The rods and cones of the vertebrate eye lie next to one another in approximately the same plane, forming a relatively flat retina lining part of the interior of the eye. In contrast, although the retinulae of the eye of *Dugesia tigrina* tend to be so oriented that their longitudinal axes are approximately normal to the nearest portion of the inner surface of the pigment cup, this orientation is far from consistent. In addition, the retinulae do not seem to lie in a single plane, but rather are distributed throughout the eye at varying distances from the inner surface of the pigment cup. Further, the fine structure of the planarian retinula differs markedly from that of the vertebrate rod or cone as described by several investigators (De Robertis and Lasansky, 1958; Sjöstrand, 1953; Wolken, 1958). Especially significant is the difference in orientation of the lamellar component, which is transverse in vertebrate receptors, but longitudinal in the planarian retinulae.

Summary

1. Anterior portions of *Dugesia tigrina* were prepared for electron microscopic examination by fixing in osmium tetroxide buffered at pH 7.2, embedding in methacrylate polymer, and sectioning at 0.025 μ.

2. The rhabdome of the retinula is composed of longitudinally oriented lamellae whose distal portions are differentiated into bulb-like swellings bearing smaller lamellae. The "middle region" of the retinula contains lamellae that appear, in some instances, to extend into the rhabdome.

3. Little analogy can be drawn between the fine structure of the retinula of the planarian eye and that of the rods and cones of the vertebrate eye.

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STUDIES OF EARLY CLEAVAGE IN THE SURF CLAM, SPISULA SOLIDISSIMA, USING METHYLENE BLUE AND TOLUIDINE BLUE AS VITAL STAINS[1]

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In 1942 Iida described the behavior of certain particles stainable with neutral red in living eggs of a Japanese sea urchin. He found the particles to be one-half to one micron in diameter, to gather specifically into the regions of the asters during cleavage and to be distributed to the daughter cells by the mitotic spindle. In addition, he noted that the particles often moved very rapidly (several microns per second), but that their movement, especially after the establishment of the asters, was restricted to a direction radial to the centrosome. He also noted that particles would move both toward and away from the centrosome, and that of two neighboring particles, one might move and the other not. He thought that the particles were attached to astral rays and that the particle movement indicated intermittent growth (with periods of retraction) of the astral fibers. He compared both the form and activity of the aster to those of a radiolarian, centrosome representing the body of the radiolarian and astral fibers its filopodia.

Independently, Pasteels (1955, 1958), Pasteels and Mulnard (1957), Dalcq (1957) and Mulnard (1958) described particles stainable with basic dyes such as toluidine blue in living eggs of molluscs, annelids, echinoderms and ascidians, which at cleavage showed a behavior similar to, if not identical with, that first described by Iida (1942). Dalcq (1954) showed that rat eggs probably possess similar particles. The more recent work was considerably more complete and traced the origin and distribution of the particles from fertilization to later cleavage stages.

In addition, histochemical work indicated that acid phosphatase and acid mucopolysaccharides (the latter having been suggested by the fact that the particles stain metachromatically in the living egg) showed a distribution during cleavage similar to that shown by the particles in the living egg. Further, two types of particles were described, α-mitochondria not astrally located, appearing in the early fertilized egg, and β-mitochondria, appearing just prior to mitosis and showing the astral localization. Evidence was adduced to support the concept that the β-mitochondria were themselves not directly stainable, but became stained with the transfer of some substance to them from the α-mitochondria.

[1] This work was partially supported by grants from the American Cancer Society and from the National Science Foundation, and was completed in part while the author was at the Department of Anatomy, College of Medicine, University of Illinois.

[2] We wish to dedicate this paper to the memory of Dr. Victor Schechter who was our first teacher in Biology and who contributed much to present knowledge of the properties of the egg of *Spisula solidissima*.

The work to be reported below was started in 1956 without knowledge of these antecedent results and took as its starting point, observations on vital staining of invertebrate eggs reported in Worley and Worley (1943) and Worley (1944a, 1944b). In this work, methylene blue was used as a vital stain and a number of molluscan and other invertebrate eggs were used as material. Certain bodies, called "Golgi" bodies by the above workers, were seen in the cytoplasm after staining, and an elaborate cycle of growth, fractionation and re-growth of these bodies during cleavage was described. This cycle was thought to be correlated to the synthesis and elaboration of lipid and protein yolk.

Our interest in this work stemmed from observations made in the course of electron microscope studies of developing eggs of the surf clam, *Spisula solidissima* (Rebhun, 1956a, 1956b). Cytoplasmic bodies considered to fulfill the criteria for yolk nuclei were there described and it was hypothesized that "heavy" protein yolk was manufactured in them, although the evidence was weak and mostly by analogy to observations and conclusions concerning yolk nuclei found in the classical literature (see, *e.g.*, Wilson, 1925). The yolk nuclei appeared to us to resemble the "Golgi" bodies of the Worleys (Worley and Worley, 1943), especially in the lamellibranch *Mytilus*, whose egg resembles that of *Spisula* in many respects. This resemblance, coupled with the suggestions of yolk synthesis claimed for each body, led us to the hypothesis that the two descriptions were, indeed, concerned with only one object (Rebhun, 1956b). This hypothesis, which led to the work described below, was, however, negated at our next opportunity to study living, stained eggs; *i.e.*, yolk nuclei are visible as refractile bodies in living eggs but never stain with methylene blue. During the course of these studies, however, we found that the particles stainable with methylene blue undergo the localization changes during cleavage described for neutral red particles in sea urchins by Iida (1942).

After this paper was accepted for publication, the author became aware of two papers by Kojima (1959a, 1959b) in which particles stainable with toluidine blue, neutral red, etc., were shown to exhibit the same behavior as those described below. In addition, Kojima showed the particles to be involved in the process of cell division. This work is discussed in a later section.

The following paper is an expansion of results already presented in preliminary reports (Rebhun, 1957, 1958).

Materials and Methods

Oocytes were removed from ripe ovaries of the surf clam, *Spisula solidissima*, and washed following the procedure described in Allen (1953). They were then stained by either of two different procedures: that of Worley and Worley (1943), used throughout the major part of this work, and that of Pasteels (1955), used after we became aware of the latter's observations. In addition, some experiments were done with neutral red following Kojima (1959a).

In the method of Worley and Worley (1943), which we will call method I, eggs were stained by leaving them on a sea table at 19°–23° C. for ½ hour in a solution of 1 part methylene blue per 1,000,000 parts sea water, ½ hour in 1 part methylene blue per 500,000 parts sea water, 1 hour in 1 part methylene blue in 250,000 parts sea water, and finally, 1 hour in 1 part methylene blue in 125,000

parts sea water. Since there is variability in staining capacity in different batches of eggs (and with different lots of dye), the above schedule was somewhat varied in terms of staining time and final dye concentration used. However, a concentration of 1 part methylene blue per 125,000 parts of sea water was not exceeded. The eggs were then thoroughly washed by gentle centrifugation in fresh filtered sea water. The eggs of *Spisula* are very hardy (Schechter, 1941) and this long sojourn in dye solution does not appear to cause injury to the eggs. This may be judged by the fact that they cleave with the same frequency and at the same rate as control unstained eggs from the same batch, and that they develop for the same length of time on the sea table (namely, about three weeks), reaching the same final stage.

Eggs stained as above will be called "lightly" stained eggs. Their cytoplasm contains many small (about ¼ to ½ micron) particles, each heavily stained. If eggs are left in the highest concentrations of dye for 1 to 2 hours longer than described above, the particles appear to increase in size (but not number) until they are 1 to 2 microns in diameter, without, however, showing any dilution in color intensity. Such eggs will be called "heavily" stained eggs. Azure A and Azure B were also used in this procedure and yielded similar results, except that developmental anomalies were more frequent with the Azures than in unstained controls. These dyes give more intense staining, and concentrations of 1 part dye per 250,000 parts sea water were not exceeded with them.

The above method is obviously suited only to unfertilized eggs because of the extensive staining periods, and, therefore, for many purposes, the method of Pasteels (1955) was used. We will call this method II. Solutions of toluidine blue at concentrations of about 1 part dye per 100,000 parts sea water were used (this is about one-fifth the concentration used by Pasteels, 1955), and the eggs were allowed to remain in such solutions for from 2 to 5 minutes. The eggs were then removed and washed thoroughly by repeated centrifugation with fresh filtered sea water in a small hand centrifuge. Concentrations of toluidine blue of 1 or more parts dye in 50,000 parts sea water will stain the thin jelly coat and the vitelline membrane, reduce the fertilization percentage, often induce some of the eggs to develop parthenogenetically, and cause the appearance of angular and elongate particles in the cytoplasm which may be crystalline aggregates of dye. At the lower concentration of 1 part toluidine blue in 100,000 parts sea water, eggs stained when unfertilized will subsequently show no difference in fertilization percentage, cleavage rate and length of developmental period on the sea table, when compared to "sibling" controls. Similarly, eggs stained with the lower dye concentration at any time during the mitotic cycle compare closely with controls in the above characteristics.

In some experiments "mitochondrial" techniques were used. The Nadi reaction was used according to Ries (1937). Zinc-free Janus Green B (kindly given to us by Dr. D. P. Costello) solutions at a concentration of 1 part dye in 50,000 parts sea water were used to stain eggs at various times in the mitotic cycle. Finally, the NBT method of Nachlas *et al.* (1957) was tried. Although this latter technique did not yield consistent results as far as appearance of eggs from different batches is concerned, it did induce interesting anomalies in cleavage which will be reported at a later date.

Photomicrographs were taken through a Leitz Ortholux microscope with Microibso attachment and Leica camera using Kodak Microfile film and D-19 developer. Over 2000 feet of time-lapse movies were made with Plus X and Tri-X 16 mm. movie film of fertilized, stained eggs at many periods from fertilization to fifth cleavage. The movie equipment used consisted of a Bolex movie camera and Samenco movie control box. The camera was supported above the microscope and was driven at rates of 1 frame per second, or 1 frame per 2 seconds by a relay actuated by the control. The light is on constantly with the Samenco control for speeds as rapid as the above. Films were analyzed visually by timing events from a screen.

In some experiments, stained eggs were centrifuged in a small high speed electric centrifuge, the speed of which was altered with a Variac. The centrifuge was calibrated with a stroboscopic lamp (Strobotac). Eggs were centrifuged against isotonic sucrose (0.95 molal) in Pyrex tubes (Kopac, 1955). Complete stratification could be obtained with 1½ to 4 minute spins at 8000 g (depending on the batch of eggs), but various regimes were tried on a given sample of eggs, *e.g.,* 1 minute at 2000 g, 1 minute at 5000 g, and 2 minutes at 8000 g. This was considered to lessen the chance of accidental trapping of particles, *e.g.,* above the nucleus. A more complete description of centrifugation techniques will be reported in the observations section.

Observations with several phase systems and the Baker interference microscope (using both shearing and double focus systems) were made on normal eggs at various times in the cleavage cycle. To help alleviate the halo and scattering effects due to refractile granules in the cytoplasm, Dr. Keith Ross and the author attempted to find an immersion medium (Ross, 1954) which would allow cleavage to proceed normally and which would match the average refractive index of the egg cytoplasm. We used bovine serum albumin, Armour fraction V in sea water, gum arabic in sea water, and a high molecular weight polyglucose, "Ficol," dissolved in sea water. Concentrated solutions were made and dialysed against sea water in a refrigerator overnight. Eggs were immersed in this medium (or dilutions thereof) on slides and were then observed.

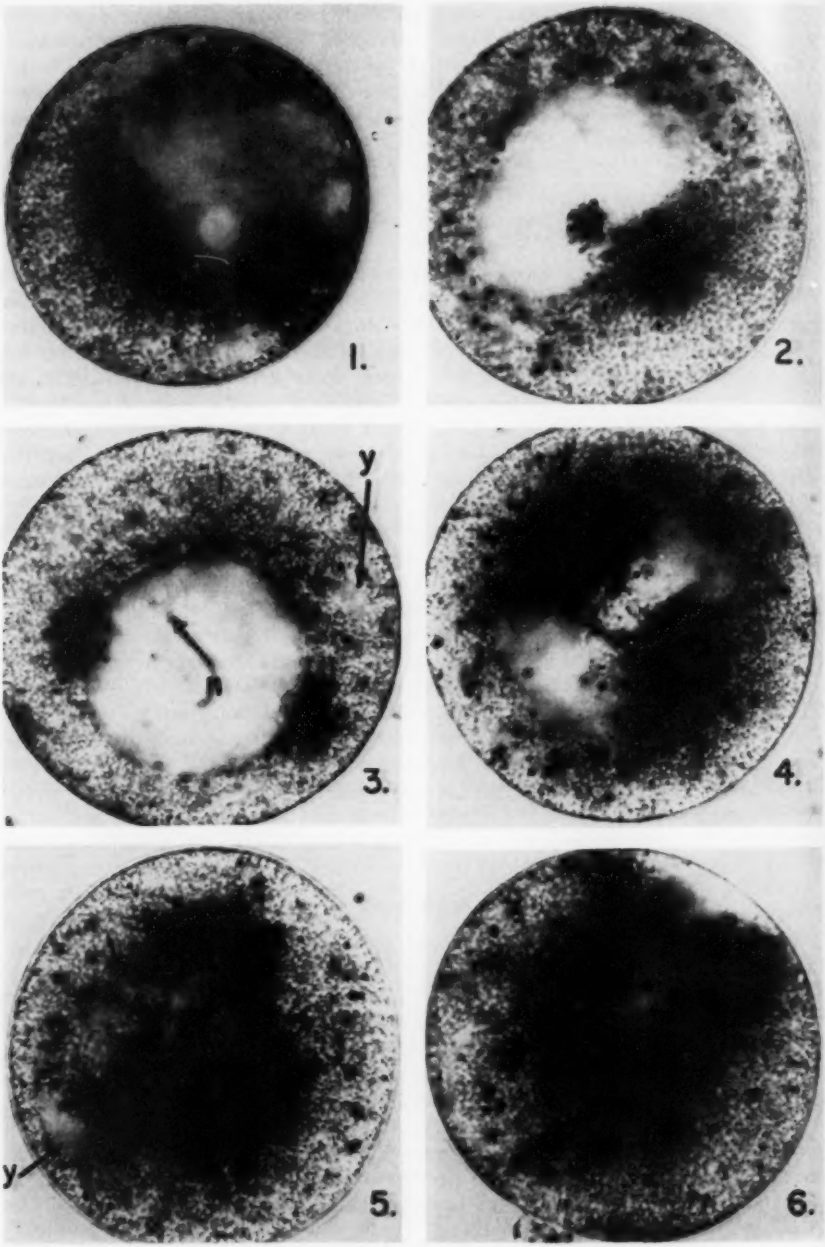
A complete report of our electron microscopic techniques will be made at a later date, although some of the results already obtained will be mentioned below.

Observations

I. *Unfertilized eggs:*

a. *Method I*

As mentioned above, small stained particles appear in eggs after several hours in solutions of methylene blue. With increased staining time the particles grow from about ¼ to ½ micron up to 1 to 2 microns in diameter. This does not appear to cause a decrease in the light absorption of the bound dye as might be expected if the dye were being diluted in a swelling vesicle. The particles appear dark whether the egg is lightly or heavily stained, and in fact, the larger particles appear darker. When eggs are removed from the staining solutions, by repeated washing in sea water, the particles do not increase further in size. Sim-



FIGURES 1–6.

ilarly, fertilization of eggs in staining solutions appears to prevent further increase in the size of the particles even though the dye is still present in the sea water.

Eggs left in staining solutions for 6 to 8 hours begin to show the formation of compound bodies with several particles aggregated into rows or around clear areas ("vacuoles") of cytoplasm 3 to 5 microns in diameter. Occasionally, very large spherical bodies up to 10 microns or more may be seen, which stain heavily with dye. We believe these compound bodies to be artifacts produced in the continued presence of dye. Yolk nuclei are visible in unstained and stained eggs (*e.g.*, see Figure 3), but, as mentioned in the introduction, never take up dye although a stained particle or two may appear within the interior of the spherical type of yolk nucleus (Rebhun, 1956b).

b. *Method II*

After short-period staining with toluidine blue, very small particles (¼ to ½ micron in diameter) appear in the egg and are uniformly distributed throughout the cytoplasm in most eggs. However, in some eggs, there appears to be a greater localization in the cytoplasmic region nearer to the nucleus. The particles take a reddish hue; *i.e.*, they stain metachromatically. The outermost layer of cytoplasm (about 1 to 2 microns thick) just beneath the vitelline membrane contains a set of particles which are somewhat elongated and are about 1 micron in over-all length although much variability in size exists. With the staining regime of method II, these cortical granules stain light blue, that is, non-metachromatically (in methylene blue solutions the granules stain light green). The two types of stained particles are thus easily distinguished by size, color, and location.

II. *Fertilized eggs:*

a. *General*

Particles in lightly stained and heavily stained eggs (method I) (taken out of dye solutions before compound bodies formed) and those stained by method II showed approximately the same behavior during fertilization and cleavage. However, until the stage of syngamy there is much more "diffuse" coloration (metachromatic) with method II than method I, which tends to obscure the events in the early stages. Most of the photomicrographs and movies, therefore, were taken

FIGURE 1. A lightly centrifuged egg with methylene blue stained particles accumulating just centrifugal to the nucleus.

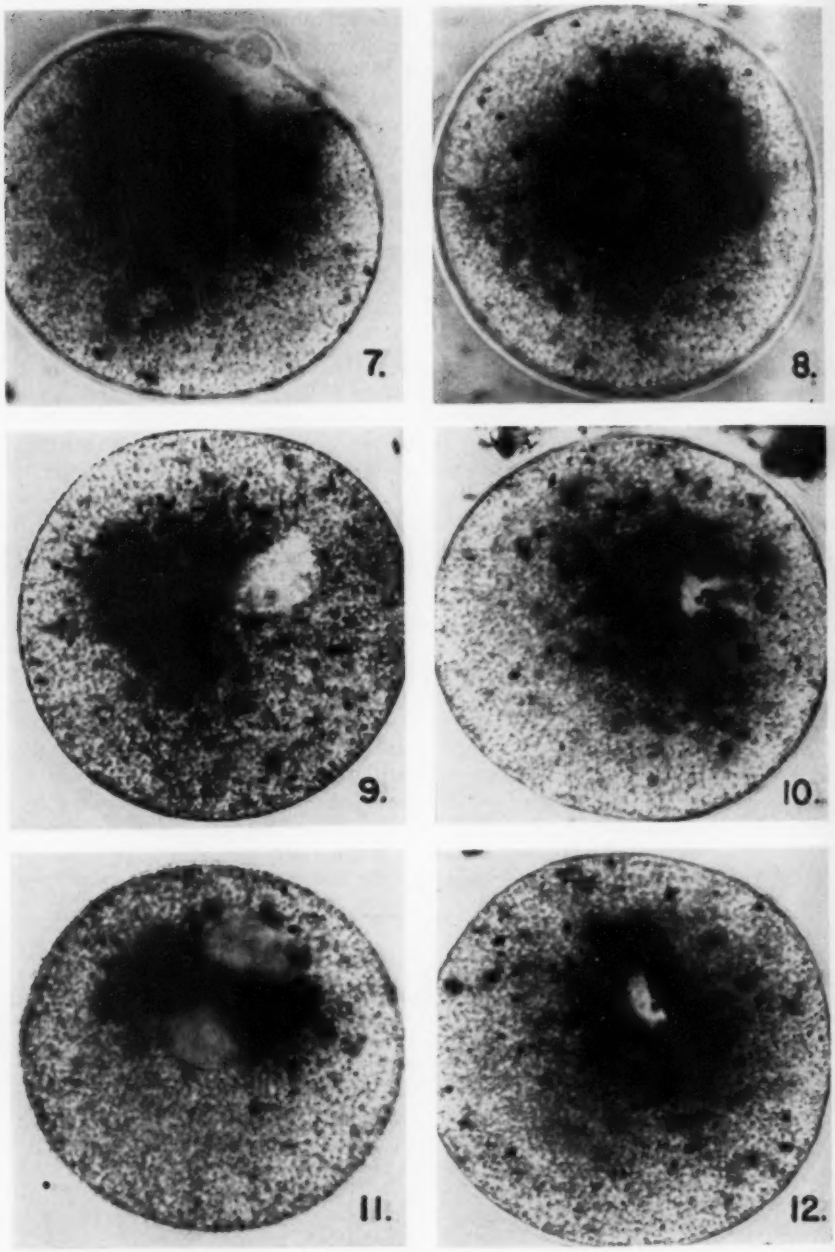
FIGURE 2. A parthogenetically activated, centrifuged egg. The aster is expanding into the nucleus.

FIGURE 3. A fertilized methylene blue stained egg. Many of the particles are gathered about the egg centrosomes, and astral rays are just beginning to indent the nuclear surface. About 8 minutes after fertilization. N = nucleolus, y = yolk nucleus.

FIGURE 4. At about 13 minutes after fertilization the astral rays have bridged the nucleoplasm and the spindle is being established. Note the masses of nucleoplasm at either side of the spindle.

FIGURE 5. At about 18 minutes the spindle is complete and begins its peripheral migration. The "excess" nucleoplasm has become intermingled with the cytoplasm.

FIGURE 6. At about 25 minutes the peripheral position of the spindle is established. Note the clear peripheral area. This is reminiscent of that seen later for first and second cleavage spindles.



FIGURES 7–12.

with heavily stained eggs which lack this "diffuse" coloration. Unfortunately, these eggs appear to be more sensitive to the light (or heat) of the microscope lamp than lightly stained eggs or unstained controls (despite heat filters and water cells) and movies of them cannot usually be taken for longer than 15 minutes. However, heavily stained eggs develop perfectly well on the sea table so that slides can be made as needed from a batch fertilized at one time.

b. *Formation of the first polar body spindle*

At about 6 to 8 minutes after fertilization, asters may be seen in the cytoplasm of the eggs, adjacent to the nuclear membrane: these may appear facing each other across the germinal vesicle or may be nearer to each other along the nuclear surface. A minute or two later the nuclear surface appears to soften, the nucleus actually enlarging (see Figure 3). The asters also begin to enlarge and the rod-like astral fibers facing the nucleus appear to push their way into the nucleus through its softened surface (Fig. 4). They elongate at the rate of about 1 micron every 2 to 3 seconds, occasionally pushing refractile (not necessarily stained) granules ahead of them from the cytoplasm into the nucleus. The cytoplasmic granules accompanying the nuclear invasion by the asters form a bridge of cytoplasmic material between the two asters (Fig. 4). In some eggs the stained particles begin to show a specific movement toward the astral centers. This is developed to a variable extent in different eggs at this time but is often quite definite. As the spindle (first polar body spindle) forms between the asters, the particles migrate more and more into the astral regions. The spindle elongates after its formation and at about 15 minutes after fertilization begins to move from the center of the egg toward the surface where the first polar body is given off (Figs. 5, 6).

When the spindle first forms, two large clear areas of nucleoplasm can be seen on either side of it (Fig. 4). They may be unequal in size, depending on the positions of the asters just before the nuclear membrane disappears. When the spindle begins its peripheral migration this nucleoplasm becomes increasingly mingled with the cytoplasm and finally can no longer be distinguished as a separate

FIGURE 7. The first polar body is formed by 31 minutes and the egg still has its oblate spheroid shape. Most of the particles are aggregated about the spindle or are, at least, in the animal hemisphere.

FIGURE 8. An optical section of the first polar body spindle perpendicular to its axis. The particles are distributed relatively uniformly. Note that many of them are aggregated into larger masses. Although difficult to photograph, these masses appear to be aggregates of smaller particles when studied visually.

FIGURE 9. The male pronucleus has not completely rounded out yet but is near the female pronucleus. About 50 minutes after fertilization.

FIGURE 10. A view along the line connecting the pronuclear centers reveals the particles lying approximately in a plane tangent to the two pronuclei at their point of contact (see Figure 11). The group of particles may be arranged in a crescent as here, or may form a complete ring. Fifty-three minutes after fertilization.

FIGURE 11. Looking at the pronuclei in a direction perpendicular to that of Figure 10 reveals the as yet intact pronuclei and the ring of particles in the plane tangent to the pronuclei. Same time as Figure 11.

FIGURE 12. The ring of particles begins to separate into two groups which will outline the asters of first cleavage. This process begins between 53 and 54 minutes after fertilization at 21° C.

entity (Fig. 5). Although some of the nuclear material may contribute to the spindle, the majority of it can be seen to mix with the cytoplasm.

From the time the asters first become visible, some of the stained particles migrate toward them (always excluding a zone about 4 to 5 microns in diameter centered on the centriole). This migration increases in intensity so that by the time the first polar body is given off at about 30 minutes, most of the particles are at least in the animal hemisphere, if not directly applied to the central aster and spindle (Figs. 6, 7). Some, but not all, of these particles move with great rapidity (several microns per second) and may appear to shoot quickly into the aster in time-lapse movies.

Many of the particles aggregate into what appear to be grape-like masses oriented radially along the astral rays (Figs. 6–8). This does not appear to be merely due to mechanical jostling since such aggregates may be seen in areas devoid of astral rays. When the spindle is first formed the stained particles outline each aster about equally. However, after the peripheral location of the spindle is established, the particles about the peripheral aster migrate to the sides of the spindle so that it is now outlined by a cup-shaped set of particles (Figs. 5 and 6). An optical section of the spindle and particles, perpendicular to the axis of the spindle, appears in Figure 8.

c. *Formation of first and second polar bodies*

At about 27 minutes after fertilization the egg begins to elongate in a direction at right angles to the animal-vegetal axis so as to become an approximate oblate spheroid (Fig. 7). The first polar body is given off at about 30 minutes (at 21° C.) and is accompanied by what appears to be a shortening, and a movement, of the spindle partly into the polar body (see Conklin, 1902, for a similar phenomenon in *Crepidula*). One or two stained particles may move into the polar body. The egg then rounds up.

Ten minutes later the second polar body is formed after a change in egg shape similar to that which occurred in first polar body formation. Throughout this period more and more of the laggard stained particles have been moving towards the spindle area and by now very few can be found elsewhere in the egg. A tight, organized mass of these particles exists around the second polar body spindle and central aster, and within this mass one can see, by careful focussing, the groups of particles forming grape-like masses.

Again, it must be emphasized that these observations can be made in eggs stained by method I or method II, although they are easier to make using method I because of the "diffuse" stain with method II.

d. *Formation of the pronuclei and their subsequent migrations*

A few minutes after second polar body formation the female pronucleus can be seen with the attendant mass of stained particles. These may completely surround it or may be gathered at one pole (pointing essentially toward the egg center). Whatever the initial distribution, however, ultimately the particles gather at the central pole of the female pronucleus.

The earliest stages of male pronucleus formation have not been seen but at

about the time that the female pronucleus becomes visible, the male pronucleus is already present and at this time appears as a clear body smaller than the female pronucleus and shaped like a short, very thick exclamation mark pointing toward the egg interior (Fig. 9). At the "period" of the exclamation mark is the center of the sperm aster which soon radiates throughout the cell. The male pronucleus increases in size and becomes spherical and about the same size as the female pronucleus.

The pronuclei have moved toward each other and by now the stained particles form a ring or crescent on the side of the female pronucleus facing the male pronucleus. The two pronuclei soon come into contact. A line drawn through their centers runs parallel to the animal-vegetal axis in the most frequent cases. The ring of particles is bisected by a plane which is tangent to the pronuclei (and thus perpendicular to the animal-vegetal axis). For views along and perpendicular to the animal-vegetal axis, see Figures 10 and 11, respectively.

It will be noticed in the above description that the particles stayed with the female pronucleus and did not move toward the male aster. This has been seen invariably in these eggs with both staining methods. However, in polyspermic eggs some particles may be seen to move into the sperm asters, usually with great rapidity (about a micron per second or so), although the bulk of particles remain with the female pronucleus.

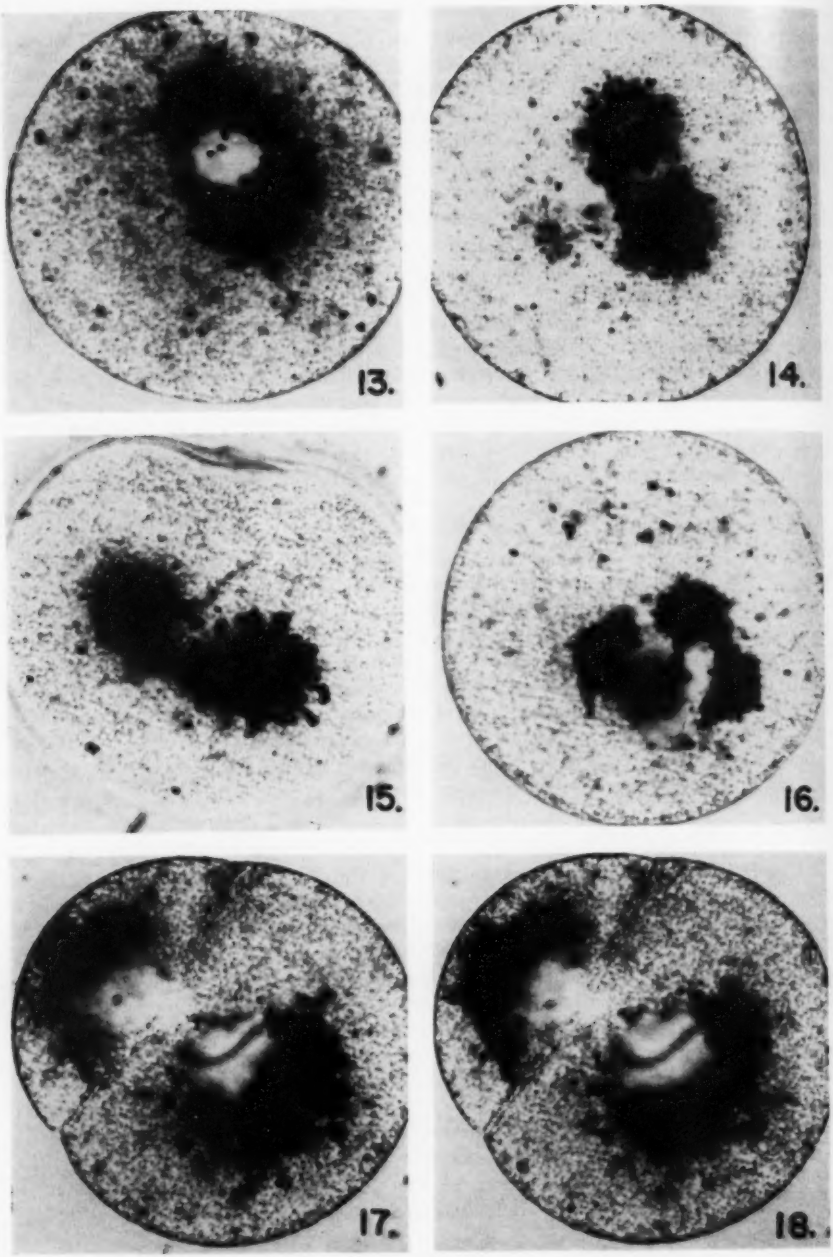
Preliminary studies have been made on parthenogenetic eggs stained with methylene blue and stimulated with KCl (see Allen, 1953). The events up to second polar body formation appear to be identical to those in the normal fertilized egg. However, a female pronucleus does not form in such eggs, although several small clear vesicles do, and, although observation of their relation to the particles is difficult, it is likely that the particles associate randomly with the vesicles. It would appear that the vesicles are similar to the karyomeres which form just prior to blastomere nucleus formation in first cleavage (see below). Although vesicle and chromosome counts were not made, it appears likely that each chromosome forms a karyomere and that, in parthenogenetic eggs, fusion of the karyomere to form a nucleus (pronucleus) does not occur.

e. *Formation of the first cleavage spindle*

The two pronuclei are now in contact and the nuclear membranes soon break down, liberating the nuclear contents without forming a true fusion nucleus. Just prior to this, the ring of stained particles divides into two half rings when seen along the animal-vegetal axis, and the half rings soon round up into partial spheres surrounding each centrosome. (See Figures 12 and 13 for two views of the same egg, about 20 seconds apart and just before nuclear membrane breakdown.) In compressed eggs it can again be seen that the particles are excluded from a region 4 to 5 microns in diameter around the centriole.

When viewed in a direction perpendicular to the animal-vegetal axis the mass of stained particles appears as a bar of material tangent to the two pronuclei (Fig. 11). This soon separates into two masses, corresponding to the division of the ring seen from the perpendicular direction.

The spindle forms in the center of the egg and remains there for about 3 minutes (Fig. 14). It is then translated along its axis to the periphery of the egg



FIGURES 13–18.

just prior to cleavage. In time-lapse movies where a frame is taken each 2 seconds, this movement looks very rapid since the whole process takes only about 45 seconds in life. The spindle is now about 30 microns in length (the egg is about 60 microns in diameter) and its axis is perpendicular to the animal-vegetal axis (Fig. 15). In the majority of eggs it appears (with accurate counts not taken) that the number of stained particles about the peripheral aster is smaller than that around the central one (for a similar phenomenon in second cleavage, see Figure 23). Although the maneuvers of the stained particles in polyspermy have not yet been studied in detail, the end result has been seen many times. The stained particles appear to divide among the asters present in approximately equal numbers so that the multipolar spindles are neatly outlined at their astral apices (Fig. 16).

f. *Spindle rocking and first cleavage*

A detailed examination of the peripheral aster at this stage reveals that a cone of clear cytoplasm free of granules of any type exists from the centrosome to the egg surface (a distance of about 2 to 3 microns) (see Figure 23 for the same phenomena in second cleavage). The stained particles surround the asters, being excluded from the peripheral area just mentioned, the spindle itself and the centrosome.

Almost immediately after the spindle translates to the periphery it begins a peculiar, regular rocking motion. Although this was first seen in time-lapse movies of stained eggs, it may be seen as well in unstained eggs by visual observation. The half-period is about 30 seconds and from 4 to 8 half-periods are completed before the motion stops. The motion is one in which the central aster remains fixed and the peripheral one moves through an arc of about 30 degrees. By close observation of the clear cone between the peripheral aster and the surface, it can be seen that this clear region moves with the spindle and therefore appears to slide to and fro beneath the cell surface (Figures 20 to 22 show the rocking phenomena for second cleavage).

The movement stops just before spindle and cell elongation occurs. The cell elongates now, in a direction parallel to the spindle axis, and so forms a prolate spheroid with the spindle on its axis. Furrow formation starts at about this time and usually advances from the region of the polar bodies (animal pole) first. The plane of the furrow bisects the axis of the spindle and because of the peripheral location of the spindle, two unequal blastomeres are formed, the large CD and small AB blastomere. The stained particles outline the asters beautifully and, as

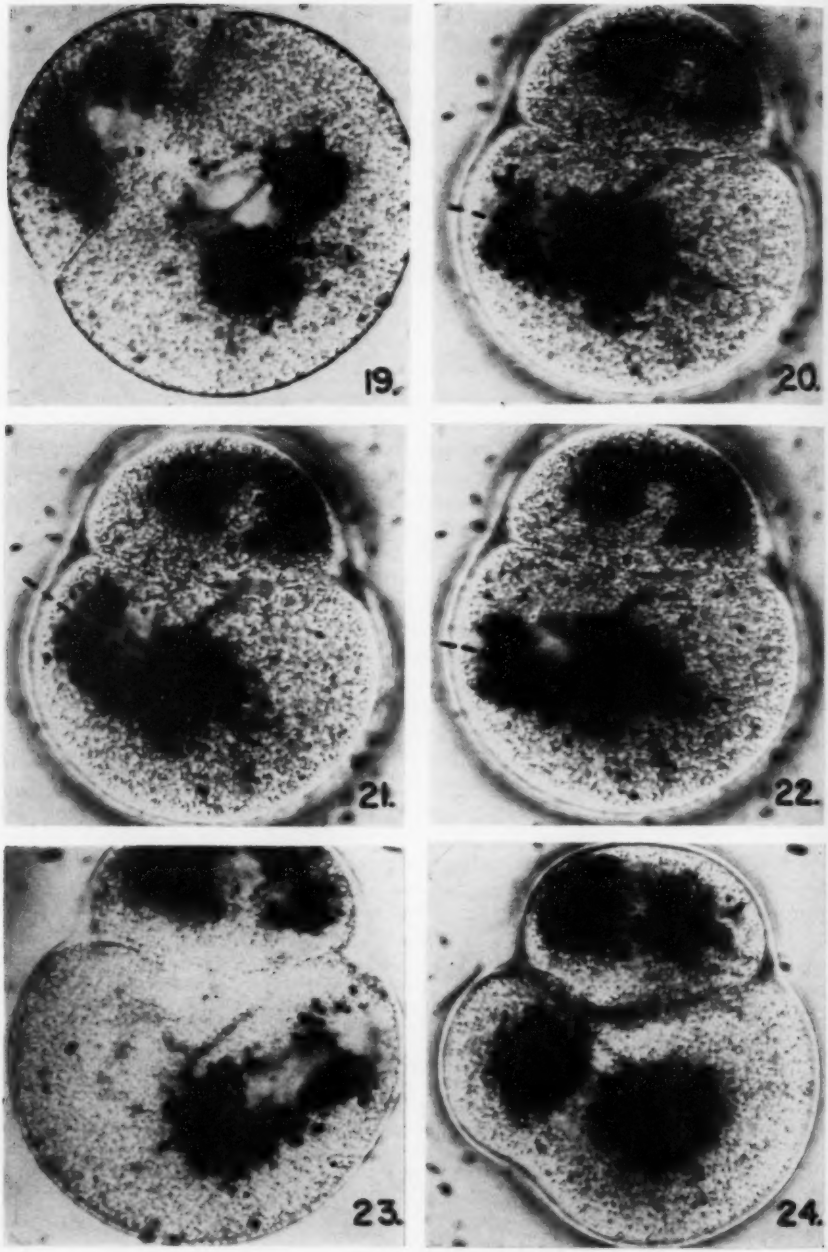
FIGURE 13. This view is of the same egg as in Figure 12, but taken approximately 20 seconds after it, and shows the beginning of the formation of the central concentration of the methylene blue stained particles.

FIGURE 14. The asters are well formed and the spindle now begins to elongate. Note the similarity of Figure 14 with Figure 4.

FIGURE 15. At about 60 minutes the first cleavage furrow starts to form, first at the animal pole. The egg has elongated in the direction parallel to the axis of the spindle. Note the radial arrangement of the clumps of particles in the aster and the "hollow" center it possesses.

FIGURE 16. Four groups of astrosomes (one slightly out of focus) indicate the ends of a tetrapolar spindle in this polyspermic egg.

FIGURES 17–18. See Figure 19.



FIGURES 19–24.

can be seen from Figure 15, almost all have localized in the asters near the centrosomes at this time.

The blastomeres round up again after the cleavage furrow has gone through. The polar bodies remain at the animal pole, beneath the vitelline membrane.

In abnormal cases arising either in eggs which have been stained by method I long enough to form compound bodies or in eggs which have been damaged by prolonged exposure to the microscope lamp, the rocking motion may continue longer (12–20 half-periods) and may attain angles up to 45°. In these cases the motion then gradually subsides with no cleavage following. On the other hand, perfectly good cleavage can occur without spindle translation to the periphery or spindle rocking. In these cases the spindle remains central in the egg and cleavage yields two equal sized blastomeres. Such cases are rare, however, and are probably anomalies in terms of later development.

In addition to the above observations, fertilized eggs previously untreated with dye were stained by method II at first cleavage. In all cases, we observed a pattern similar to that seen in eggs stained before fertilization: that is, an astral location of the metachromatic particles. In some batches of eggs, there were few, if any, discrete stained particles anywhere else in the cell. However, in most other batches of eggs, many stained particles were seen scattered throughout the cytoplasm (appearing on casual observation as a "diffuse" stain), although a heavier astral concentration always existed. The stain in the astral regions was always light compared to that which would be seen at this time in eggs which had been stained before or just after fertilization, so that eggs often had to be compressed to see astral localization.

The further maneuvers of particles stained at this late date are identical in nature to those stained before fertilization.

g. *Interphase and prophase of second cleavage division*

Nuclear reconstitution takes place by the formation and fusion of small karyomeres ("chromosomal vesicles") as can be seen most clearly from electron micrographs. This same process occurs in *Chaetopterus, Arbacia* (Gross *et al.,* 1958) and *Fundulus* (Richards, 1917) and is probably of quite general occurrence in molluscs (Raven, 1958). The stained particles begin to gather at one pole of the blastomere nucleus, primarily that facing the periphery, although sometimes a little to one side. The particles are rarely found on the surface of the central hemisphere of the blastomere nucleus (Fig. 17). After formation of the cap of stained particles, one sees remarkable movements in some of them in time-lapse

FIGURE 19. Figures 17 to 19 are taken of the same egg about 20 seconds apart and indicate (a) the peripheral location of the astrosomes on the blastomere nuclei and (b) the separation of the particles into two groups just prior to nuclear membrane breakdown. Unfortunately, a piece of lint traverses the image of the nucleus in the CD blastomere.

FIGURES 20–22. Microphotographs of the same egg taken about 20 seconds apart. This shows the 30° excursion of the spindle in a half rotation. Note the fixed central aster.

FIGURE 23. The oscillation in this egg has stopped and the pre-cleavage elongation has occurred. Note the peripheral cone of clear cytoplasm, the radial arrangement of the astrosomes and the smaller number of particles about the peripheral aster.

FIGURE 24. The cleavage furrow is practically completed at about 75 minutes in the CD blastomere. The AB blastomere lags by about 1 minute.

movies or by direct visual use of the light microscope. They may move very rapidly (1 to 2 microns per second) in a radial direction, shuttling back and forth in straight lines between the nucleus and the periphery. In time-lapse movies they may appear to jump discontinuously.

This movement subsides before the next division, which occurs 15 minutes after first cleavage. The prelude to this cleavage is a division of the mass of stained particles into two masses which then appear to slide over the intact nuclear surface, ultimately coming to lie at opposite poles of the nucleus (Figures 17 to 19 show this process in one egg: the pictures are taken 20 seconds apart). This undoubtedly corresponds to prophase of the second division, but the nuclear events cannot be seen easily enough to identify early stages by chromosome morphology. The division of the mass of stained particles into two sub-masses occurs about 1 minute earlier in the large CD blastomere than in the small AB blastomere, which correlates with the 1-minute difference in cleavage times (Allen, 1953).

The particles forming the two masses are arranged so as to indicate the poles of the second cleavage spindle. It can then be seen that the axis of this spindle in each blastomere makes an angle of 90° with that of the spindle of first cleavage. On the other hand, the axes of the spindles of the two blastomeres in some, but definitely not all eggs, are tipped at about 30° to each other as can be seen when looking along a line parallel to the axis of the first cleavage spindle (see Conklin, 1902, for a similar observation in *Crepidula*).

h. *Second cleavage*

Second cleavage in the AB blastomere is easily described and presents no unusual aspects. The two groups of stained particles outline the asters and show the same characteristics described for first cleavage asters, *i.e.*, aggregation of particles into the aster, movement of particles in a direction radial to the astral center, etc. (Fig. 19).

The spindle for the CD blastomere forms approximately in the center of the blastomere. It then shows a sudden movement to the periphery and toward the AB blastomere (which has not yet divided). The movement takes about 45 seconds and results in the spindle axis, in its new position, making an angle of about 30° with the axis of the spindle in the position in which it first formed. Except for this tipping, the motion is reminiscent of the spindle translation seen in first cleavage. The resemblance is further strengthened by the occurrence of a rocking motion on the part of this spindle of about the same period, amplitude, and form as in first cleavage (Figures 20 to 22 show a period in the rocking motion, the photographs having been taken about 20 seconds apart). By the same form is meant the fact that it is the peripheral aster which moves, the whole spindle rocking about the fixed central aster. Again in conformity with the events in first cleavage, a cone of clear cytoplasm exists between the peripheral aster and the egg surface, and the peripheral aster with its cone appears to slide to and fro under the surface (Fig. 23). Finally, the number of stained particles near the peripheral aster is smaller than that near the central aster (Fig. 23). It is thus clear that cleavage of the CD blastomere is very similar in course to that of the whole egg in first cleavage.

The rocking motion stops after 4 to 8 half-periods and in its final position the

spindle is tipped at about 30° to the animal-vegetal axis, that is, it has returned to the position from which it started its rocking motion (Fig. 23). The cell elongates approximately parallel to the spindle and the cleavage furrow then begins to pinch in, as in first cleavage, from the animal pole first. The result is a large D blastomere and a small C blastomere (Fig. 24).

As in first cleavage, in some cases the spindle fails to migrate to the periphery. The resulting blastomeres are then of equal size. In such cases irregular rocking motions of the spindle sometimes occur.

Later cleavages have not been studied in detail but the same process of division of the stained particles into two masses, outlining of the asters and, finally, reconstitution of a single mass on the nuclear surface has been followed as far as fifth cleavage and there is no reason to suppose it stops here. In addition, in two sequences of a time-lapse movie of third cleavage, the spindle of the D blastomere (but not that of the others) was seen to form in the center of the cell, move to the periphery, and show the same spindle rocking movements as seen in the whole egg in first cleavage and the CD blastomere in second cleavage.

It should be pointed out that the number of particles stained appears to remain the same from the unfertilized egg on, so that they appear to be diluted in each blastomere in each division. This by no means implies that new particles do not arise in each division but simply that if such particles arise it is probably not through division of previously stained ones.

i. *Centrifuged eggs*

Staining experiments with centrifuged eggs were carried out primarily with toluidine blue, although neutral red (Kojima, 1959a) was used in some runs, the results being essentially identical. The eggs were generally stratified at 8000 g for periods of 1½ to 4 minutes depending upon the batch of eggs (clams from different populations behave quite differently with respect to stratification times). In addition, a limited number of eggs were partially stratified at 4380 g for 1 minute at approximately the first polar body stage and at first cleavage.

Four types of experiments were run: (1) eggs were stained before fertilization and then stratified at various periods through second cleavage; (2) eggs were fertilized, stained for several minutes starting at 5 minutes after fertilization and then stratified at various periods through second cleavage; (3) eggs were fertilized and then stained for from 2 to 5 minutes immediately before centrifugation at various times through second cleavage; (4) eggs were fertilized, stratified at various times through second cleavage and then stained, usually for not more than 3 minutes, to avoid major redistribution of particles before observation.

Before describing the results of the above experiments, several considerations must be mentioned. First, the stratified egg begins to redistribute quickly, so that observations must be rapid. Thus, large numbers of eggs must be surveyed under low power and estimations of the location of stain in "typical" cases made in a matter of minutes. Cases may then be selected for longer study at high power (oil immersion). Second, eggs destain rapidly if oxygen is excluded from the preparation as is inevitable when compressed eggs are used. Third, in many cases the vitelline membrane and jelly coat stain heavily with dye (metachromatically) so that it is very difficult to decide whether a "diffuse" stain is, indeed,

intra- or extracytoplasmic. Fourth, the cortical granules stain blue and interfere with observations in the centrifugal portions of eggs (see below). Fifth, many refractive yolk granules in the centrifugal parts of stratified eggs place considerable restrictions on accurate observation in this zone. In assessing the results of the experiments, the reader must bear the above considerations in mind.

After germinal vesicle breakdown, the unstained, stratified *Spisula* egg shows five constant layers, with some overlap in the more centrifugal ones: most centripetally, a cap of lipid granules; next, a clear layer; then, a mitochondrial layer; a yolk layer; and, finally, most centrifugally, a layer of cortical granules (these do not break down on fertilization in the *Spisula* egg). In addition, pronuclei may be found immediately below the lipid cap after second polar body formation, and a spindle after syngamy, although the latter is by no means easy to see.

1) *Behavior of particles in eggs with a germinal vesicle (unfertilized eggs to eggs about 10 minutes after fertilization.)* The results of experiments on such eggs are the same whether staining is carried out after or before centrifugation. In all cases the majority of the metachromatic particles can be seen in a narrow layer centrifugal to the germinal vesicle (a layer identified as a mitochondrial layer with the electron microscope) (see Figure 1). Usually, a small number of the particles can be seen in the lipid cap and in the centrifugal yolk area. In about 2 to 3 per cent of the cases, many of the cortical granules accumulate at the centrifugal pole. The metachromatic particles, as seen with dye concentrations of 1 part toluidine blue in 100,000 parts sea water, are small (about ¼ to ½ micron in diameter) and stain heavily. With higher dye concentrations, many particles appear as stained vesicles more than a micron across. This is especially true with neutral red.

Some of the unfertilized eggs are parthenogenetically stimulated by the sucrose "pycnotic" barrier. In such cases particles are closely associated with the asters formed (see Figure 2).

2) *General behavior of particles in eggs after germinal breakdown.* After germinal vesicle breakdown to at least second cleavage, the metachromatic particles can be found in two locations: a layer at the centripetal end of the clear zone, and a layer at the centrifugal end of the yolk area. These general locations can be seen in all four types of staining experiments performed (*i.e.*, with relation of staining to centrifugation times), and, indeed, the localizations appear identical, although, in general, the closer the observation time is to the staining time, the *lighter* does the stain appear. The particles in both widely separated locations appear to have the same size and staining characteristics as the particles in uncentrifuged eggs (remember, however, the difficulties of observation in the yolk area). With high dye concentrations, 1 part or more toluidine blue in 50,000 parts sea water, the particles in the centripetal layer may be considerably larger than those in the centrifugal layer.

In addition, when centrifuging eggs at the stages in which polar bodies are given off or after syngamy, one sees two other phenomena in most if not all eggs; one, there is a thin, clear zone separating the centripetal layer of particles from the lipid cap (this is not present when pronuclei have formed); two, one often sees very tight knots of particles in this centripetal zone of particles. If one follows these tight aggregates for 10 to 15 minutes, one gradually sees the mass grow

larger, the radial arrangement of grape-like masses of stained particles form and, in general, the development of all the activity characteristic of asters. It would appear that the centripetal particles represent the β-particles of Pasteels (1955), by both their location in the centrifuged egg and their behavior relative to the asters. It is clear that these particles are directly stainable, since they were seen at all times in the mitotic cycle, at the most 4 minutes after the beginning of staining and, in one experiment, at 2½ minutes after the beginning of staining (see Discussion).

The results of staining centrifuged eggs at various times in the cleavage cycle differ somewhat from those with unstratified eggs so stained. If, at first cleavage, one compares an unstratified egg stained before fertilization with one stained at this stage, one finds in most batches of eggs an extensive "diffuse" stain in the latter, not found in eggs stained before fertilization, which may obscure the astral aggregation of metachromatic particles unless the eggs are compressed. In addition, the astrally aggregated particles appear to stain more lightly in eggs stained at first cleavage. The eggs stained in these two ways thus look different in gross appearance, one egg clear, the other "diffusely" stained. The centrifuged eggs look much more similar in this regard, although the eggs stained after centrifugation at first cleavage stain less heavily. In watching the latter, it can be seen that the centripetal particles (β-granules) increase in color intensity with time. This increase in intensity of β-granule staining, coupled with a destaining of the vitelline membrane and jelly coat, probably contributes to the differences in "diffuse" staining seen in the unstratified eggs.

It is clear that after germinal vesicle breakdown two groups of stained particles can be seen. We saw that this division of particles into two groups with the centrifuge occurred in eggs which had been stained before fertilization and then stratified at first cleavage. However, such eggs when observed before stratification appear to be almost completely devoid of particles anywhere in the egg except in the astral regions, and it, thus, is important to know where the two groups of stained particles come from. To get at this problem we centrifuged eggs lightly at 4380 g for 1 minute at the first polar body stage and at first cleavage. This partially stratifies the egg (a definite hyaline zone is not present, although lipid, yolk, and cortical granule layers are). In eggs stained before fertilization, the spindles with attendant stained particles were very clear. In many such eggs no metachromatic particles could be seen in the centrifugal yolk area, although this was not universally true. If eggs were stratified first and then stained, the particles in the asters were seen but were lighter in stain than in the previous case. In addition, particles were seen in the centrifugal zone, but it was impossible to determine if there were more or fewer than in the first experiment. In eggs from these same batches, centrifuged at the higher speeds and longer times discussed earlier, the two groups of particles, centripetal and centrifugal, were clearly seen.

j. *Other staining methods*

Our results with both the Nadi reaction and Janus Green B staining can be simply stated: at all stages in mitosis the stain appears only in the mitochondrial layer in centrifuged eggs, and in non-centrifuged eggs we find only a uniform distribution of stain in the cytoplasm external to the spindle (for a different result,

see Dalcq *et al.*, 1956). This result agrees with that which would be predicted from our electron micrographs of eggs at various mitotic stages, since in these micrographs, no specific localization of mitochondria can be seen in uncentrifuged eggs at any stage of mitosis. In centrifuged eggs, the electron microscope shows mitochondria to be gathered primarily into a layer just centripetal to the yolk (both in fertilized and unfertilized eggs), although they can occasionally be seen in the lipid and yolk layers (see also Pasteels *et al.*, 1958).

Discussion

The events of early cleavage in *Spisula*, which we have set forth above, are similar in many details to those described in other lamellibranch eggs such as *Unio* (Lillie, 1901), *Mactra* (Kostanecki, 1904), and *Barnea* (Pasteels, 1930). A discussion of all aspects of the cytology of these stages (which can be found in Raven, 1958) would be out of place in the present paper and we shall concentrate only on some of the more puzzling problems which these observations raise.

A problem which fits the latter category is that of spindle translation and oscillation in first cleavage, first reported by Lillie (1901) in the fresh-water clam, *Unio*. Lillie found no morphological structure revealed by his technique which could account for the precision and movement which the first cleavage spindle possesses. He felt, however, that there must be some invisible (but by no means mystical) organization of the cytoplasm which imposes its influence upon the spindle, and that this is part of that more general organization which determines the mosaic character of the egg. That he observed the spindle oscillation as well as translation seems clear from his statement that "the position of the spindle is controlled through the cytoplasm as a needle in a magnetic field oscillates until equilibrium is attained" (Lillie, 1901 : page 255).

Spindle movements in the gastropod, *Crepidula*, were described by Conklin (1902, 1912) and were ascribed by him to the influence of cytoplasmic currents. The peripheral migration of the first polar body spindle was thought to be due to an axially directed vegetal-to-animal current which moved the spindle to the animal pole. The characteristic alternation of spindle directions in third and later cleavages in *Crepidula* (and presumably in other *Spiralia*) was considered to be due to currents which themselves altered direction with cleavage in late anaphase. Experimental interference with those currents appeared to stop cleavage (Conklin, 1938).

A cortical influence on spindle displacements appears from the work of Pasteels (1930, 1931) on *Barnea candida*, and, indeed, Raven (1958) feels that the primary influence originates in the cortex. This influence presumably affects the spindle through the cytoplasm, possibly through an influence on cytoplasmic currents.

Our own observations on spindle movements, both visually (with the microscope) and in time-lapse movies, make it clear that cytoplasmic displacements and spindle movements are correlated events. However, the dissection of cause from effect in these phenomena is by no means obvious. Indeed, in many time-lapse movie sequences of spindle movement, the rapidity of its inception and apparent directedness of its trajectory leave a definite impression of an object pulled, rather than pushed, through the cytoplasm. It is not impossible that a morphological

connection of a temporary nature may attach the spindle to the egg cortex, and that contraction of this connection is the event which displaces the spindle toward the cortex.

Some indirect evidence for such a view can be cited. First, the egg cytoplasm is capable of rapid localized contractions. These can be seen in *Spisula* eggs which become amoeboid after membrane removal with alkaline isotonic NaCl (similar amoeboid phenomena in eggs have been reported by Lillie, 1902, and Monroy, 1948, in annelids; by Pasteels, 1930, and Kostanecki, 1904, in molluscs; and by Harvey, 1938, and Moser, 1940, in echinoderms). Very rapid "twitches" may occur in some parts of the eggs while other parts appear quite fluid (*i.e.*, are flowing). Second, a close relation appears to exist between the cortical granules and the spindle ends. This is a normal phenomenon in later (*i.e.*, third and on) cleavages and will be reported in detail in a later publication. In addition, in eggs treated with alkaline isotonic NaCl, as early as first cleavage cortical granules (as distinguished by size, shape and staining properties) may appear tightly aggregated about a single pole of the spindle (which in these cases does not migrate) (for such a relation in *Mactra*, see Kostanecki, 1904), indicating connections of the spindle pole with the cortex which allows the granules to be pulled to the pole under the experimental conditions described above. Such connections (if they exist) need be no more permanent than astral rays (which they may indeed be). Finally, if such connections consisted of local gelated regions of cytoplasm (fibers, rods), their movements relative to the surrounding cytoplasm might, indeed, have been described as cytoplasmic currents. All in all, one need not be bound to the idea of a fluid current as being the motive force in spindle movement.

Existence of particles

In any study using the techniques here discussed a primary question is the existence of the particles prior to treatment. The controversies concerning the origin and existence of the "vacuome" (Hovasse, 1956) visualized after neutral red staining make any such staining techniques guilty, until proven innocent, of the production of the particles by the techniques themselves. This is the more so, since a type of reaction to injury in the egg is the formation of vacuoles (Heilbrunn, 1956).

We have briefly discussed the vital staining experiments of Pasteels, Mulnard, and Dalcq in the introduction. In addition to this work with the living cell, these authors used the Gomori acid phosphatase technique and the Alcian blue technique for acid mucopolysaccharides, and were able to show, in several invertebrates, that acid phosphatase and acid mucopolysaccharides in the egg showed the same localization changes as those undergone by the dye particles in the vitally stained egg (Pasteels and Mulnard, 1957; Pasteels, 1958; Mulnard, 1958). We have confirmed some of these observations with *Spisula* and will report them at a later date. These histochemical results indicate that, at least, there is a moiety (or "plasm") of the egg which predates any treatment with dye, and which follows the centrosomes, although it does not prove that this moiety is particulate. We will discuss this problem after first comparing our results with those of the Belgian school.

Comparison of the present work with that of Pasteels, Mulnard, and Dalcq

We have already indicated that the above authors feel that there exist in the egg two types of particles: a small, lightly staining metachromatic one, called the α-granule (or α-mitochondrion) which can be present and formed at any time in the cleavage cycle, and a larger, deeply staining one which appears in the cell only at certain specific periods in the "life" cycle of the egg (although it may exist before it can be stained). In the molluscs, *Barnea* and *Gryphea* (Pasteels and Mulnard, 1957), the sea urchins, *Psammechinus miliaris* (Pasteels, 1955) and *Paracentrotus lividus* (Pasteels, 1958), and the ascidian, *Ascidiella aspersa* (Dalcq *et al.*, 1956; Dalcq, 1957), this period is primarily that during which copulation of the pronuclei is beginning to occur, although some particles may be formed at the time of the second maturation division. In *Chaetopterus pergamentaceus* (Mulnard, 1958) the particles begin to appear at the first maturation division.

Several bits of evidence are adduced by the Belgian workers for the existence of two particles, the most telling being the so-called indirect staining of the β-granules. That is, the observation that eggs stained at cleavage show only a diffuse light granular stain (α-particles), the astral location only occurring at the next cleavage and then in particles larger and more darkly staining than the first. In *Chaetopterus*, however (Mulnard, 1958), there appears to be some question as to whether the β-particles are not directly stainable since in several cases Mulnard reports their appearance after staining "à un stade où les granules β existent déjà en grande nombre" (Mulnard, 1958, page 657), and, therefore, their direct stainability. He feels, however, that this is probably due to their rapid formation during the five-minute staining period he used and during which time material may be transferred to the β-granules from α-granules.

Two further bits of supporting evidence for the two-particle idea come from vital staining experiments and from histochemical tests. Thus, Pasteels (1958) claims that in *Paracentrotus* (the one exceptional species, so far) the α-granules do not stain with the acid phosphatase technique (although the β-granules do). In addition, the α-granules stain with neutral red but the β-granules do not, and thus neutral red particles do not show an astral location. This last result should, however, be contrasted to the neutral red results obtained by Iida (1942) in a Japanese species of sea urchin (species not given) and by Kojima (1959a) in three species of sea urchins.

It is clear from the above discussion that the same basic phenomena have been described by ourselves and the Belgian group. It is equally clear, however, that differences exist. In view of the variation in results which that group have themselves reported in different species, it can be assumed that at least a part of our differing observations can be traced to different materials.

Our own vital staining work supports the idea that the particles pre-exist the treatment with dye. Thus, in all cases staining of eggs immediately before or immediately after centrifugation at any point in the cleavage cycle gives essentially the same result (keeping in mind the precautions noted in the section on observations). Indeed, if the dye were causing the formation of particles (or vacuoles) from a pre-existing, but non-particulate, moiety of the egg, one would expect the vacuoles to have a different relative specific gravity from this "precursor" and, therefore, to show a different centrifugal behavior from it, which the experiments

discussed in Observations, section II i, show to be false. This change in relative specific gravity is, however, what occurs in the eggs heavily stained with methylene blue, where the particles are deliberately caused to swell, and where they centrifuge to the upper stratum of the yolk layer.

A question of great interest is that of the number of types of stained particles present. Our centrifugation experiments described earlier indicate, in agreement with the work of the Belgian group, that after germinal vesicle breakdown, two groups of particles are present, one group centrifuging to the centripetal part of the hyaline layer (for more detail, see observations) and one, to the centrifugal end of the yolk layer. These particles appear to be about the same size (they are too small for very accurate measurement) and to stain with approximately the same intensity if examined 10 to 15 minutes after staining. The location of the particles in the stratified egg seems to agree with the locations of the α- and β-granules of the Belgian group. The fact that the centripetal particles migrate into the asters in redistributing eggs leaves little doubt of this identification. We may, therefore, identify our centripetal particles with the β-granules of the Belgian group, and the centrifugal particles with their α-granules. With this identification it is clear that a fundamental difference of observation exists between the work reported here and that of the Belgian school, namely, that we find the β-granules to be stainable directly and to be present and identifiable at all points in the cleavage cycle after germinal vesicle breakdown. This is supported by direct observation on compressed eggs stained with low dye concentrations at appropriate times in the cleavage cycle, although, as was mentioned in the section on observations, the difficulties due to "diffuse" staining must be carefully overcome. In addition, the observable fact that β-granules increase in depth of stain with time must be taken into account.

A further difference between our results and those of the Belgian group (although one fraught with some observational difficulties as previously discussed) derives from the results of centrifuging eggs at first cleavage, which were stained before fertilization (or soon after). Eggs at first cleavage show essentially no stained particles elsewhere in the egg other than in the asters. In lightly centrifuged eggs one finds many cases in which metachromatic particles are found only in the (centripetally located) asters. However, such eggs stratified with the higher forces show the two groups of particles, α- and β-granules. It would appear, therefore, that both α- and β-granules, in *Spisula*, show an astral aggregation, which is maintained under low, but disrupted under high centrifugal forces.

To reiterate, then, in *Spisula* eggs one finds two sets of particles, both directly stainable with toluidine blue (or neutral red, etc.) and both of which show the migration into the asters. What the relationship between these particles is (and to what degree the centrifugal behavior of the particles represents a real difference in their nature) we cannot say from our observations. Our work neither lends support nor in any way detracts from the idea of the Belgian groups that the α-granules are precursors to the β-granules. However, in *Spisula*, if this is so, the process of β-granule formation must be continuous throughout the cleavage cycle, with the β-granules so formed, directly stainable.

Direct confirmation of the existence of the particles in the untreated living cell would still be highly desirable. Dalcq *et al.* (1956) report that particles

showing the behavior under discussion can be seen, with the phase microscope, in the living egg. However, using both phase and interference microscopy, with and without the immersion media earlier discussed, we have not been able to confirm this observation, although it is true that occasionally dark particles (in dark contrast phase), or particles of a slightly different hue from other particles in the cell (in interference microscopy), appear near the centrosomes in living, unstained eggs. Unfortunately, the presence of many highly refractile granules in eggs makes such observations unreliable, since even in compressed eggs halos make detailed observations difficult in phase microscopy. Reversed color effects from particles not in the image plane make interference microscope images also unreliable in this case. In addition, the contrast in a phase microscope is a function of the optical path difference between an object and its surround (Bennett *et al.*, 1951), and, since the average refractive index of the cytoplasm outside the asters and spindle is different from that of the asters and spindle themselves (Ross and Rebhun, unpublished experiments; Mitchison and Swann, 1953), it would be expected that particles near the asters would show some contrast difference with those elsewhere. These disappointing results may simply mean that the optical path difference between these particles and the cytoplasm and that between other cell particles and the cytoplasm is about the same and the particles are therefore not distinguishable by this property alone.

A similar disappointing result has so far been obtained by us with the electron microscope. Our work (Rebhun, 1958) and that of Pasteels *et al.* (1958) seem definitely to remove mitochondria from consideration as candidates for the stained particles, reversing a previously stated suggestion (Pasteels and Mulnard, 1957; Dalcq *et al.*, 1956).[3] Pasteels *et al.* (1958) feel that in centrifuged eggs of the sea urchin, *Paracentrotus lividus*, they can see "Golgi" bodies in the layer to which the acid phosphatase positive, metachromatic β-granules go. Unfortunately, they present no electron micrographs to support this claim. In addition, they do not see these bodies specifically associated with the asters. In our own electron micrographs of *Spisula* we can occasionally see at least two different types of particles possibly associated with the asters, one of which looks superficially like Golgi bodies in having closely packed concentric lamellae surrounding a vacuole. However, this may merely indicate a complex lipid cortex such as occurs in the methylene blue, brilliant cresyl blue and Nile blue staining phospholipid particles in pulmonate snail neurons (Chou, 1957; Ross and Chou, 1957). Indeed, in electron micrographs of such neurons, bodies, such as described above in electron micrographs of *Spisula* eggs, do occur (Ross, personal communication). The second

[3] I would like to clear up a misunderstanding concerning these results which apparently occurred during a conversation with Dr. Mulnard and which I discovered only upon reading his paper (1958). My position concerning the existence of the particles is there reported as one of scepticism which, it is claimed, stems from electron micrographs of heavily stained eggs (method I). These micrographs do, in fact, show vesicular bodies not seen in unstained eggs, with single or double membranes outside and vesicles or "cristae"-like (Palade, 1953) objects within. It was clear to me, however, as discussed in this paper, that these large bodies arise from the prolonged action of dye upon a pre-existing substratum. Among the possible particles which might be involved as substratum, I considered at that time, a particle with the morphology of mitochondria but with different enzymatic properties, but realized this to be very hypothetical. I now reject this possibility. I have never thought these particles to be degenerated, "ordinary" mitochondria as stated by Mulnard (1958).

type of particle resembles the multivesicular bodies in rat eggs (Sotelo and Porter, 1959). All in all, the electron microscope is disappointingly vague on the questions relevant here. We shall, however, report these results in detail at a later date.

Some observations which may have a bearing on the problem of the general existence of the particles other cells, are those of Bloom *et al.* (1955, Fig. 2) on newt heart fibroblasts. The movies from which this paper is taken show many dark particles (called "fat droplets" by the authors) which migrate to the centriole region of the cell in prophase and show a distribution in division very similar to that described above in eggs. Indeed, there appears to be enhanced movement of many of the particles and an aggregation into grape-like masses during division, similar to that described in eggs. Histochemical studies of these cells, however, have not been reported.

Finally, Holt (1957) showed that esterase and acid phosphatase in mitoses, after formalin fixation, in regenerating rat liver showed the same localization changes that the basic dyes and acid phosphatase do in eggs.

Preliminary identification of the particles

Work has already been quoted which indicates that these particles are not mitochondrial in nature. We have also indicated our attitude of constraint (not, however, negation) toward the suggestion that the β-particles are themselves Golgi bodies. The suggestion remains (see Mulnard, 1958) that the α-granules are lysosomes in the terminology of de Duve (1957), this being supported by the fact (ignoring Pasteels' (1958) negative result in *Paracentrotus* for α-granules, for the moment) that the α-granules contain acid phosphatase. The lysosome suggestion is strengthened by the observation of Holt (1957) that acid phosphatase in liver parenchymal cell mitosis shows the same behavior as our particles do in living eggs, since the lysosome concept originated from particles obtained from liver. Our preliminary work with egg homogenates indicates that the acid phosphatase of the egg is sedimentable at relatively low speeds, rather than soluble. However, we find no evidence that the activity is increased by treatment with Triton-X, aging at 37° C., freezing and thawing, or treatment with distilled water. Thus, the egg particles lack a fundamental property of lysosomes, *i.e.*, a *releasable* acid phosphatase. These results will be reported in detail at a later date.

Motion of the particles

As has been pointed out, the particles may move with great rapidity and in fixed directions. This motion is superimposed on a Brownian motion similar to that undergone by other particles in the cytoplasm. The characteristics of the motion of the stained particles are: (a) they may move with velocities up to several microns per second; (b) two particles within a few microns of each other may move rapidly in opposite directions; (c) the rapid movements are, generally, radial to the centrosome, either towards or away; (d) not all particles move rapidly at any given time, *i.e.*, some may be undergoing ordinary Brownian motion; and (e) these motions are *not* participated in by most other inclusions in the cell (the motions of cortical granules in later cleavages may be an exception). Any mechanism proposed to explain these phenomena must explain them all.

There are several types of forces which might be invoked to account for these events. For example, Bjerknes (see Schrader, 1953) published accounts of differential movement of particles relative to each other on fluids in which standing waves were set up by two pulsating spheres. The movement of the particles towards or away from the spheres depended on the phase of the vibrating spheres and the density of the particles relative to the fluid medium. This phenomenon has been used in models of anaphase separation of chromosomes (Schrader, 1953), and Pfeiffer (1956) has published accounts of supposedly pulsating centrosomes. We feel, however, that much more evidence for such standing waves or pulsating centrosomes would have to be gathered before such a mechanism could be considered as causing particle movement in eggs. In addition, the α- and β-granules appear to participate in the movement although their centrifugal behaviors, and therefore densities, are very different.

The intermittent growth of the astral fibers was postulated by Iida (1942) as causing the motion of the particles. This requires the particles to be attached to the astral rays in some way. Their normal Brownian motion, however, would seem to argue against such an attachment (although the fact that they centrifuge with the asters might support it). Also, the net direction of motion of the particles during mitosis is into the asters at a time when the asters are growing and, therefore, moving out. Finally, there is no evidence of a shortening of astral rays during their growth period as is required to pull the particles into the asters on the above model.

Cytoplasmic currents might be invoked as agents sweeping the particles into the asters. Conklin (1902) and Chambers (1917) considered the aster to be essentially composed of such streams. We feel, however, that certain observations speak against this notion, namely, the motion of some stained particles relative to nearby stationary ones, and, more particularly, their motion relative to yolk granules and mitochondria in the same region which remain stationary (apart from Brownian movement). Currents should be relatively indiscriminate in moving small particles of approximately the same size. Moreover, the motion of some of the particles is so rapid, and starts so suddenly, that it is difficult to imagine currents being involved. Finally, the same particle may be rapidly "jerked" into the aster and just as rapidly reverse its movement, although the net direction is inward as cleavage proceeds.

In connection with the last observation, we suggest the possibility of mechanical connections of some sort, possibly contractile fibers or gel streams, connecting the stained bodies to the centers. Contractile fibers of this nature have been postulated as being involved in the motion of echinochrome granules in *Arbacia* (Parpart, 1953). After fertilization, these particles move radially into the egg cortex (McClendon, 1910). In movies of this phenomenon, it is clear that the motion of echinochrome granules has exactly the same five properties as listed for the metachromatically stained particles, if we replace the word "centrosome" by the words "egg cortex" in (c) above (A. K. Parpart, personal communication).

We suggest, again, as for the case of spindle movement, that contracting gel streams, similar to those described by Allen (1955) in amoebae, might very well be involved in these motions, and that such streams might easily be described as cytoplasmic currents.

A detailed study of the relative movements of individual particles of different types at different times in the mitotic cycle, similar to the study of Hiramoto (1958) on cleaving *Clypeaster* eggs, would be most useful in deciding between some of the suggestions discussed above.

Function of the particles

Some recent work has thrown interesting light on some possible functions the particles may perform. Marsland (1958) has extensive evidence from pressure centrifugation work on premature furrowing in *Arbacia* eggs that a factor from the nucleus and one from the metachromatic particles are involved in the initiation of the cleavage furrow. This work is supported by the beautiful work of Kojima (1959a, 1959b) which indicates that halves of eggs obtained by splitting with the centrifuge, cleave or do not cleave depending upon whether they do or do not receive, during centrifugation, particles such as we have been discussing. In the sea urchins *Temnopleurus* and *Mespilia* stratified but not broken eggs contain particles in the centrifugal end and it is this end which cleaves subsequently. Other evidence was adduced by Kojima in support of the hypothesis that the particles are involved in cleavage initiation.

Summary

1. Techniques for vitally staining oocytes of *Spisula solidissima* with certain basic dyes are described.

2. Such staining reveals small particles in the cytoplasm of the egg.

3. After fertilization the particles show very specific movements and localization changes which can be described by saying that the particles follow the centrioles through cleavage.

4. Spindle movements and position changes can easily be followed and are described in detail.

5. The results are discussed in the light of related work in the literature.

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WOUND HEALING PROCESSES IN AMPUTATED MOUSE DIGITS[1]

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For obvious reasons the healing of wounds has been the object of study by many biologists and medical researchers. The literature is enormous and basic information concerning the subject can be found in many excellent reviews by Marchand (1901), Arey (1936), and Cameron (1952), and has been incorporated in text books of Histology, Maximow and Bloom (1957), Surgery, Harkins (1957) and Pathology, Robbins (1957).

Difficulties inherent in mammalian material and the desire for uniform techniques in the study of wound healing have caused the non-clinical investigations to be concentrated upon a limited variety of wounds. Studies of epidermal and dermal healing have been generally confined to skin wounds made on the backs of rats, Lindquist (1946) or on rabbit ears, Clark and Clark (1953), and Levander (1950). The important problem of bone repair and regeneration has been studied predominantly in rib or leg fractures in rabbits, Ham and Harris (1956), cats, Blaisdell and Cowan (1926) or rats, McLean and Urist (1955).

But when one considers the problem of the type of wound that occurs after amputation of an appendage it is surprising to discover a lack of fundamental information of the subject. Systematic post-amputational wound healing has heretofore been studied only in the lower vertebrates, probably because of their capacity to regenerate limbs. Studies in man have been confined to gross observations and to attempts to hasten the process of healing via skin grafts and other devices (Slocumb and Pratt, 1944). Occasional studies have appeared in the literature that dealt with amputational healing in rat (Nicholas, 1926) or sheep fetuses (Barron, 1945); however, even these biologists confined themselves to gross observations. We have been unable to find any description concerned with the histological process of post-amputational wound healing in mammals.

This paper constitutes an attempt at a systematic investigation of the histological aspects of wound healing processes occurring in amputated mouse digits. Our objectives are two-fold: first, to describe the post-amputational events that lead to simultaneous and coordinated repair of skin, connective tissue and bone; secondly, to evaluate the findings from the point of view of the student of regeneration, and to ascertain whether there are any aspects of this process that might be comparable to those seen in regenerating vertebrates.

Materials and Methods

The animals used in this study were four- to eight-week-old Swiss mice of a strain purchased from the Roscoe B. Jackson Laboratory in 1953 and maintained at this laboratory to the present time. They were fed a diet of Purina Lab Chow

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and kept at a temperature of 80° (± 2° F.) in plastic cages in groups which never exceeded seven mice per cage.

Previous attempts at a systematic histological study of post-amputational wound healing in mammals conducted at this laboratory (McIntosh, Amherst College Honor's Thesis, 1954) were confined to amputations made through the carpal, metacarpal or radius-ulna regions of the forelimb. It was found, particularly in the younger individuals, that the many bones at the amputation site were often severed at different stages of ossification, surely a complicating factor in an histological study. It is also reasonable to assume that a long bone amputated through the diaphyseal region might present a healing pattern different from the one amputated through the epiphysis. It was therefore decided to select for study the digit containing within each particular segment a single bone at a definite stage of development. The digits of the forelimb, because of their small size (roughly one millimeter in diameter), are ideally suited for an experimental and histological study.

The digits to be amputated were identified with the numbers I to V starting with the short digit which is homologous to the human thumb. The phalanges are numbered 1 to 3 from proximal to distal. Phalanx 3 is quite small and almost completely covered with a nail, so digital segments containing the longer phalanx 2 of the second, third and fourth digits of both forelimbs were selected for levels of amputation. Using the above numeration, a code system was devised to identify the amputation stump and host animal. Thus, for example, the third digit from the left forelimb of Case MCS 50, amputated through the second phalanx, was designated in our records and identified in this paper as Case MCS 50, L-2-III.

The mice were narcotized for the initial amputation with ether. Before fixation of the amputation stump, the animals were narcotized with a subcutaneous injection of veterinary Nembutal (Pentobarbital Sodium, Abbot) permitting a deeper narcosis. The doses varied from 1.8 mg. to 2.4 mg. depending upon the age, size and condition of the animal. The amputations were performed with small surgical scissors under the dissecting microscope. Due to considerable bleeding, no attempt was made to trim the bone stump to the level of the soft tissues; it is probably for this reason that in some cases a protruding bone complicated the healing processes. To avoid using an excessive number of animals, four to six digits were amputated on the same individual and these amputations were done either simultaneously or at varying intervals.

Before fixation, the digital hair was removed with a commercial depilatory (Nair). The stump of the digit was severed at the metacarpophalangeal joint and fixed in Bouin's solution for three days or longer and decalcified in Jenkin's solution for periods ranging from six to fourteen days. The embedding procedures entailed three paraffin changes and a 24-hour perfusion in the last paraffin bath at 56–58° C. All digits were sectioned at 10 micra. Difficulties in sectioning encountered were attributable to incomplete decalcification. Most sections were stained with Harris's modification of Delafields' hematoxylin and counterstained with orange G. Mallory's polychromatic stain was also used to show development of connective tissue fibers.

This investigation is based upon histological studies from 191 digits fixed at various times ranging from six hours to seven weeks after amputation.

Experimental Results

Although, as mentioned in the introduction, the role of the epidermal, dermal and bony tissues in the healing of wounds has been well studied, it was desirable to re-investigate all these processes in the healing of amputational wounds. The healing stumps were fixed and studied histologically at six-hour intervals for the

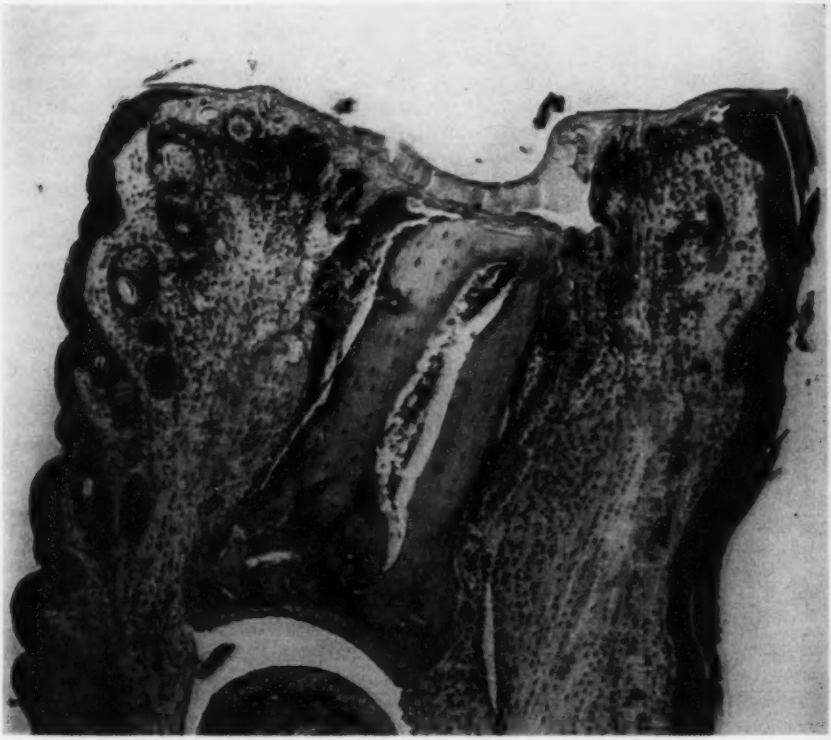

Figure 1. Photomicrograph of a sagittal section from a digit (Case MCS 52, L-2-III) fixed six hours after amputation. This case illustrates several aspects characteristic of the early wound healing process: (a) the entire surface of the cut digit is covered by a layer of blood coagulum with only a few blood cells in it; (b) beneath this coagulum can be seen large masses of white blood elements characteristic of this early inflammation phase; (c) the cut edges of skin show the beginning of epithelial migration and healing (90×).

first two days, and daily thereafter for three weeks. The healing of epidermis, dermis and bone as observed in these amputational wounds will be discussed with particular emphasis upon the unique interrelationships between these tissues.

For aid in orientation when viewing the figures, a sagittal section of a mouse digit illustrating its typical anatomy is presented in Figure 1 (Case MCS 52, L-2-III). The skin on the right side of the photomicrograph represents the volar

surface, the left side representing the dorsal surface. The palmar or volar surface of the mouse digit is characterized by the absence of hair follicles and by a thick epithelium and dermis, while the dorsal epithelium possesses many hair follicles and is thinner than the volar. When a digit is sectioned horizontally the follicle distribution permits one to determine the exact position of the section. Amputation in this case was made through the mid-diaphyseal region of phalanx 2, and it is possible to observe the retracted extensor and flexor tendons on both sides of the bone.

The early hours of wound healing after amputation are characterized, according to Arey (*op. cit.*) and Robbins (*op. cit.*) by provisional closure with a blood clot and subsequent inflammation. Six hours after amputation (Fig. 1) the wound

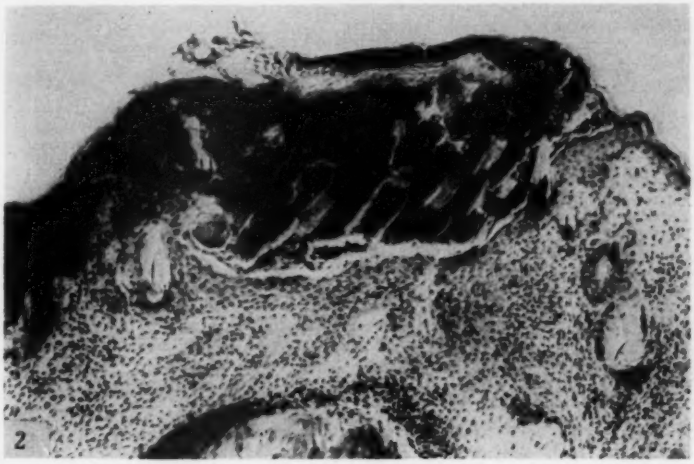

FIGURE 2. Photomicrograph of longitudinal section from a digit (Case MCS 129, L-2-III), fixed one day after amputation. A continuous layer of epithelial cells covers the amputation surface and it separates from the stump the large scab composed of clotted blood and various tissue debris. Inflammation at this stage has spread into the stump, and large masses of leukocytes have accumulated around the cut end of the bone (150×).

area is covered by a solidified blood clot beneath which polymorphonuclear leukocytes are agglomerating. By one day (Fig. 2, Case MCS 129, L-2-III), the extent of necrosis as indicated by the area of leukocytic activity has spread deeper into the tissues, particularly around the cut end of the bone. The polymorphonuclear leukocytes that characterize the early stages of inflammation begin to die off and they become extruded with part of the scab (Fig. 2). Subsequently monocytes and lymphocytes from the blood and young macrophages from the tissues identifiable in large numbers for at least three days replace the polycytes.

The first stages of epithelial migration and healing are in most aspects similar to those first described by Loeb (1898) in his classic work on epithelial wound healing. As seen in a digit fixed six hours after amputation (Fig. 3, same case as Fig. 1), the first cells to migrate derive generally from the granular layer

of the epithelium. These cells rapidly swell, elongate and begin to migrate into and over the newly formed clot. The "syncytial protoplasmic layer" which according to Loeb (*op. cit.*) covers the clot or scab cannot be confirmed from the evidence of Figure 3, and from all the other sections we have studied. Rather, we have repeatedly observed an exudate (clearly shown in Fig. 3) of the type to which Weiss in several of his papers attracts attention (see last report on the subject, Weiss, 1959). We have made no histochemical study of the constitution of this

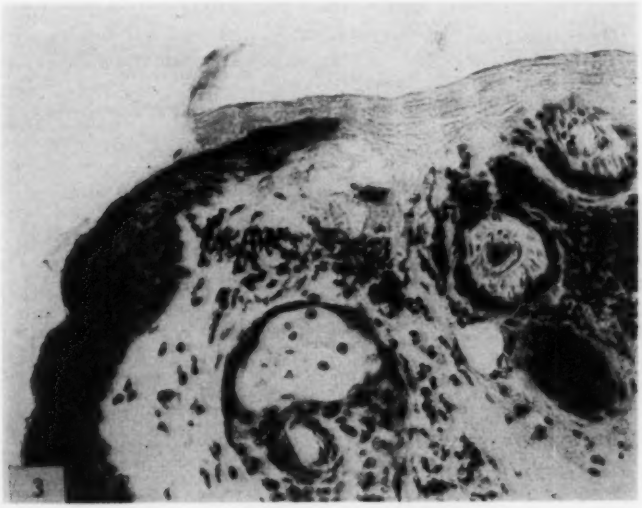

FIGURE 3. Photomicrograph of the upper left amputation site of Case MCS 52, L-2-III (shown in Fig. 1). The early migration six hours after amputation of the epithelium from the dorsal skin edge is shown. The migratory elements derive from the granular and lower horny layers of the epithelium, and the migrating wedge of cells can be seen invading the acellular exudate exhibiting fibrous structure. Beneath the clot large numbers of inflammatory leukocytes surround the hair follicles (300×).

exudate, but the photomicrograph clearly shows its orientational patterns (parallel to the amputation surface) so important for "contact guidance" (Weiss, *op. cit.*) of epithelial cells migrating over a semisolid substrate.

The migration from the basal Malpighian layer proper takes place less rapidly. At six hours these cells are just beginning to swell and to elongate, and it is not until eighteen hours after amputation that a migrating wedge of epithelium from the Malpighian layer can be observed to infiltrate the area beneath the scab and the tissue debris. Once started this migration continues more rapidly, since one day after amputation a continuous layer of epithelial cells from the Malpighian layer can be found covering the area of tissue debris underneath the scab (Fig. 2). It is, however, only exceptionally that the epithelial covering is completed within one day. As a rule epithelial healing is observed in the majority of the digits only three days after amputation (eight out of the thirteen cases studied showed

at three days a complete epithelial covering of the stump), in some other digits, undoubtedly due to protruding bone debris, this process is still further delayed. Complete epithelial repair is shown in Figure 4 (Case MCS 55, R-2-II) where the new epidermis shows early stratification. Also shown on the figure is a continued proliferation and migration of the Malpighian layer resulting in this and in other cases in a thick (12 cell layers) epithelial wedge over the cut end of the bone (see also Fig. 5, Case MCS, R-2-III). This local or general thickening of

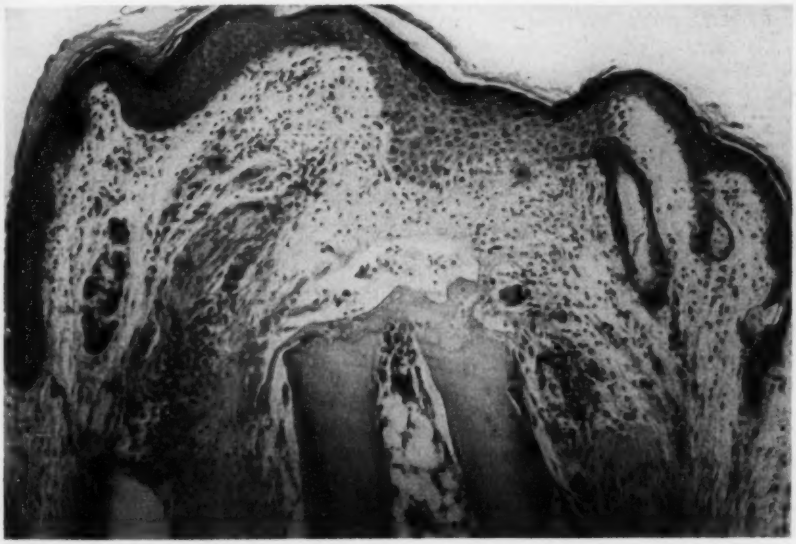

FIGURE 4. Photomicrograph of a longitudinal section from a digit (Case MCS 55, R-2-III), fixed three days after amputation and showing complete epidermal healing. Note: (a) the differentiation of epidermis into several distinct layers and the epidermal wedge with loosened cellular elements extending toward the bone; (b) between the covering epithelium and the bone are amorphous masses of clotted blood and tissue debris, while to the left of the bone masses of necrotic tissue can be seen; (c) individual fibroblasts identifiable by their spindle shaped form can be seen migrating anteriorly on both sides of the bone shaft; (d) the circular orientation of the tissues and the small area of epithelial proliferation, ahead of the phalanx reflects the contraction of the amputational wound (110×).

epidermal layers after amputation is a feature which closely resembles that described by Rose (1948) as a regular feature of epidermal growth in early regeneration in the newt.

The reconstruction of dermal tissues begins three days after amputation. Fibroblasts from surrounding connective tissue and particularly from the regions of cut tendons can be seen streaming into the wound area. They proceed to intermingle with the pool of red cells and tissue debris that is characteristically found between the cut end of the bone and the new pad of epithelium. Once started, the proliferation and migration of fibroblasts proceeds rapidly and by six days (Fig. 5) a completed cap of fibroblasts separates the epithelium from the cut end

of the bone. Seven days after amputation 13 of the 15 cases studied showed a well defined dermal pad.

Our observations of dermal healing concur with the previous descriptions of Arey (*op. cit.*) and Robbins (*op. cit.*): new blood vessels with at first indistinct walls accompanying the migrating fibroblasts become evident. Also, the first new collagenous fibers are seen at seven days in preparations with Mallory stain; consequently, the sub-dermal tissues become increasingly dense and fibrous until about two weeks after amputation when a characteristic dermal pad is found surrounding the end of the bone and the newly formed callus.

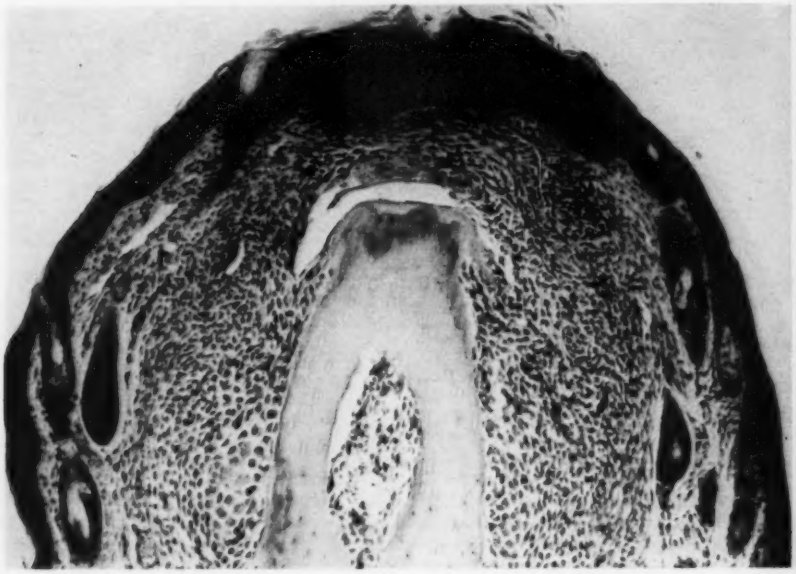

FIGURE 5. The photomicrograph of a longitudinal section from a digit (Case MCS 1, R-2-III) fixed six days after amputation shows: (a) the thick epithelial covering; (b) the connective tissue fibroblastic invasion into the amputation area resulting in a sizeable cap consisting of numerous layers of connective tissues; (c) the bursa-like cavity between this dermis and the end of the bone; (d) the extensive proliferation of periosteal elements with a large mass of cartilage cells on both sides of the bone (125 ×).

Reconstruction of bone tissue is also seen for the first time at three days, and our observations of the early stages of repair agree with the descriptions of McLean and Urist (*op. cit.*). The trauma of the operation and particularly the disturbance of vascular supply results in the death of osteocytes for varying distances from the distal end of the cut bone toward its proximal region. Empty bone lacunae such as shown in Figures 4 and 5 are represented under higher magnification in Figure 6 (Case MCS 100, R-2-III), fixed three days after amputation. The empty lacunae occupy most of the bone shaft; however, near the periosteum surviving osteocytes are discernible. The existence of surviving osteocytes at the proximal end of the

bone shaft indicates that necrosis affects only the osteocytes located within the distal area of the bone affected by amputation. We have observed that the dead bone matrix is subsequently destroyed and removed by macrophages and giant cells, while the remaining bone tissue undergoes osteoclastic reorganization, which is particularly active around six days after amputation.

Reconstruction of new bone and the formation of a callus begins about three days after amputation as a proliferation of the osteoblasts forming the endosteal and periosteal coverings of the bone (Fig. 6). Once started, the proliferation of these osteogenic cells proceeds quite rapidly and by six days (Fig. 5) the early stages of callus differentiation become noticeable. On other sections not here

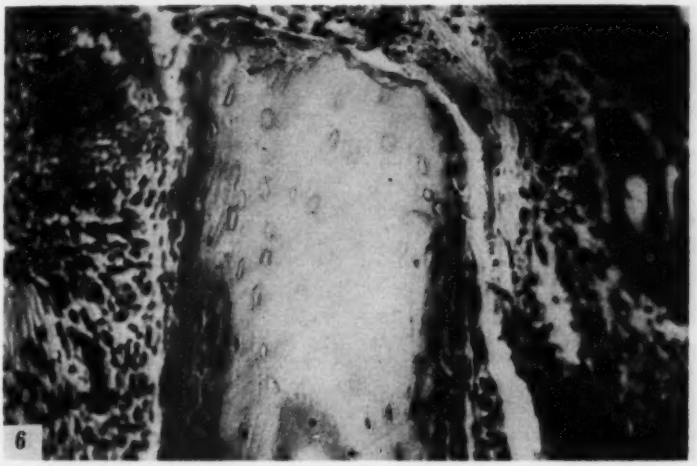

FIGURE 6. Photomicrograph of phalanx 2 from a digit (Case MCS 100, R-2-III) fixed three days after amputation showing: (a) the dead tip of the bone recognizable by the empty bone lacunae, while more proximally and toward the periosteal edges live osteocytes are visible; (b) the proliferation of the osteogenic cells in the periosteum on both sides of the bone (240 ×).

illustrated, new bone trabeculae may be seen next to the old bone tissue, while the elements farthest away from the bone retain a fibroblast-like appearance. The cells which remain in an intermediate position generally go through a cartilage cell stage which is well illustrated in Figure 5 (to the left side of the shaft).

The size and shape of the callus that is formed varies according to the level of amputation. Observations indicate that amputation through the diaphysis of the bone results in a large callus with a characteristic intermediate cartilagenous stage, while if the epiphyses are transected a much smaller callus results. At the former level new bone trabeculae originate first near the bone and then at the outer edge of the callus. The cartilage cells intermediate between these two levels either disintegrate to make room to a highly vascularized zone that closely resembles the former hematopoietic marrow cavity, or they become calcified to leave thin bone trabeculae connecting the new outer shell of the stump to the old bone.

The formation of a new callus collar around the diaphyseal shaft is best shown in a cross-section of a thirteen-day-old amputation stage (Fig. 7, Case MCS 1, L-2-III). The outermost layers of osteogenic cells appear to be the largest and most active. Between the outer layer of new bone trabeculae and the old bone shaft can be seen perpendicularly arranged trabeculae, cartilage cells, and blood elements. Not indicated in this figure, but nevertheless observable in many sections, is a final callus that is much larger and of a more amorphous appearance than that typically described for healing in fractures (Ham and Harris, *op. cit.*).

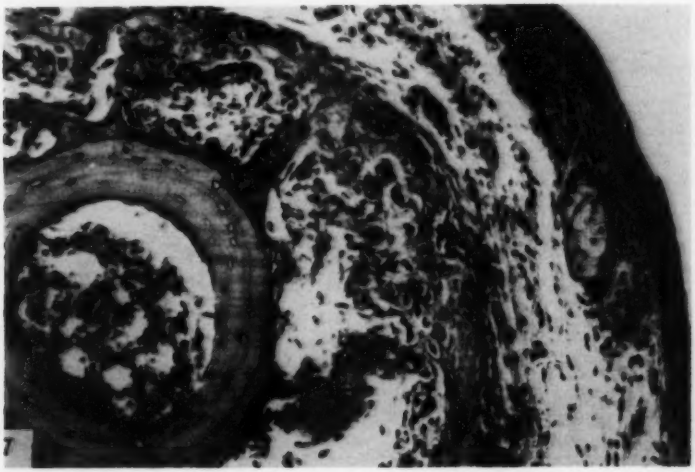

FIGURE 7. Photomicrograph of a cross-section from a digit (Case MCS 1, L-2-III) fixed thirteen days after amputation, showing the shape and extent of callus formation that follows amputation through the diaphysis. Observe the ring of new tissue formed around the old bone ring. External to the new layer of bone is a layer of large periosteal osteogenic cells. Between the peripheral region of the callus and the old bone new bone trabeculae, cartilage and blood elements are discernible (200 ×).

Amputation through and near the spongy epiphysis of the bone results in a different type of callus formation. Heavy proliferation of osteogenic elements is rare and, when found, these collect at the cut surface of the bone nearest to the diaphysis. Few cartilage cells are seen and remodeling appears to take place by osteoclasts and destruction of bone rather than by the formation of new bone trabeculae (Fig. 8, Case MCS 31, L-2-III).

Of particular interest in this and in other similar cases is the relative lack of reconstruction within the endosteal surface of the bone (Figs. 7 and 8), its main functions seeming to be confined to hemopoietic activities. Another interesting feature is the observation that the marrow cavity is invaded by connective tissue elements soon after amputation, and in these cases the processes of hematopoiesis revert to normal only after bone repair is completed.

The formation of bursa-like cavities. The aspects of post-amputational wound healing described so far concerned first the tissues immediately affected by ampu-

tation such as skin, and secondly the effects upon regions of the severed bone not directly injured by amputation. These are general reactions, encountered in any type of wound.

However, in amputational wounds of an appendicular organ such as a digit, features of healing are revealed which are peculiar and unique. Among these is the appearance of a pocket or tissue space which forms regularly over the end of the bone. At three days one observes a characteristic fluid area of necrotic debris and of free blood elements occupying the space between the cut bone and the covering epithelium (Fig. 4). With the onset of dermal healing, fibroblasts invade this area and five or six days after amputation the pool or pocket acquires a definite shape. The cavity or fluid space, similar to that seen in Figure 5 and which in many instances possesses a discrete synovial-like lining, closely resembles in histological features the bursae described by Black (1934).

Mention has been made in the literature of such an occurrence in amputated mammalian limbs: Nicholas (*op. cit.*) relates the formation of a bursa over the cut bone in a rat limb amputated in utero and fixed ninety days after birth, but no histological description was given. Nunnemacher (1939), studying the effects of partial amputation of epiphyseal cartilages in long bones of the same animal, describes and illustrates the appearance of a bursa within the connective tissues over the end of the cut bone. These observations are complemented by those of Urist, Mazet and McLean (1954) who describe the formation of a pseudo-arthroidal joint between the end of fractured bones that failed to appose. It does seem that a new bursa with a synovial-like lining is formed in amputational stumps in general, and that it is due to friction and irritation in much the same way as a pseudo-arthroidal joint is formed between the separated ends of a non-healing fracture.

Our observations differ from the above in the fact that the formation of a bursa-like cavity in amputated mouse digits was the rule rather than the exception: the fluid-filled area invariably appeared upon closure of the epidermis three days after amputation, acquired anatomical definition as a cavity in the midst of the invading fibroblasts, and then disappeared after the first post-amputational week. This last observation is in contrast to those made by the aforementioned authors in that bursal cavities here reported were transitory, while theirs were permanent.

Terminal aspects of wound healing in a mouse digit and in a frog limb

After the events described, the further post-amputational healing processes center around additional growth and thickening of the sub-dermal tissues. This latter thickening is effected by further fibrogenesis within the connective tissue, and the extreme degree of development of a pad of connective tissue in a digit fixed thirteen days after amputation is represented on Figure 8. The photomicrograph indicates that the digit was sectioned in a frontal, not a dorso-ventral plane, as the hair follicles are evenly distributed on both sides of the section. The figure shows that healing of the skin has been completed, the epidermal layers having reverted to their normal thickness; also, the capping of the end of the bone by the thick multi-layered pad of connective tissue suggests the end of growth of the cut phalanx. Observation of the dome-shaped connective tissue pad under higher magnification reveals its extensive vascular supply and also the "adult"

appearance of the blood vessels. As was explained above, bone callus formation is only slight in this case because amputation was effected near the proximal epiphyseal region; however, periosteal cells are in the process of chondrofication and some new bone trabeculae have appeared. For all intents and purposes healing has been completed and little proliferative activity remains. It is clear from

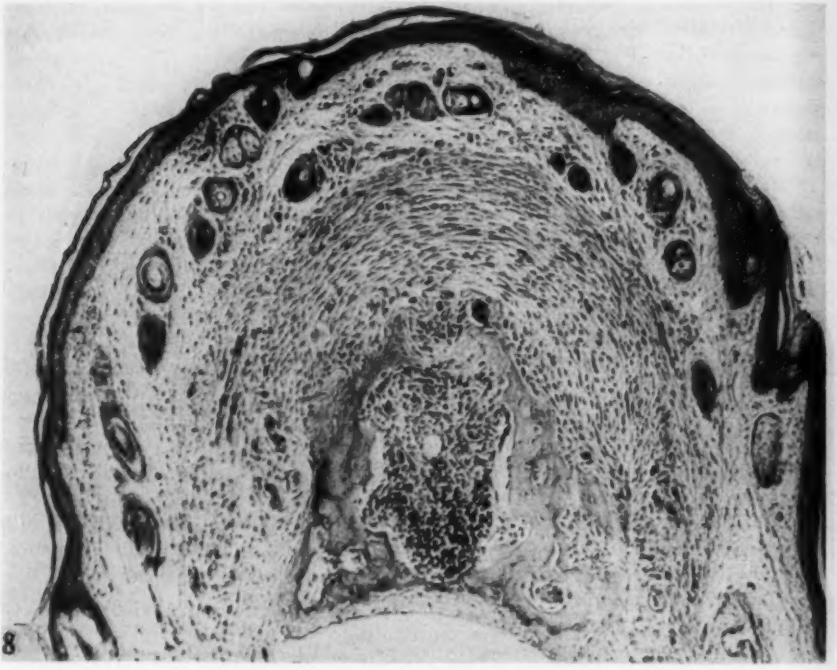

FIGURE 8. Photomicrograph of a longitudinal section from a digit (Case MCS 31, L-2-II) fixed 13 days after amputation, illustrating completion of epidermal and dermal healing. The epithelium has thinned out and has resumed a normal appearance together with the completely reconstructed dermis and sub-dermal layer containing numerous hair follicles. In addition a thick cap of concentric layers of fibroblasts has developed over the cut bone and it separates the injured tissues from the fully re-formed skin. Within the connective tissue cap numerous new blood vessels are distinguishable. Since amputation was near the epiphysis there is little callus formation (110×).

the examination of this and of similar slides from comparable post-amputational stages that no growth nor "regeneration" has occurred and that within a fortnight an amputated digital stump has reached true tissue equilibrium.

This "final" stage of wound healing observed in an amputated mammalian digit may be compared to advantage with a similarly "terminal" stage of amputational wound healing in another, also non-regenerating vertebrate, a frog. A section from a forelimb of a post-metamorphic frog (*Rana clamitans*, 4.5 cm. long

from snout to crotch), amputated through the lower arm and fixed 97 days after amputation will serve this purpose (Fig. 9). The longitudinal section from the lower forelimb shows: a completely regenerated skin as found on old amputational wounds; a cushion of sub-dermal formations including a fibroblastic pad tightly drawn over the terminal shaft of the ulna; finally, there is a well developed callus surrounding the severed shaft of the ulna.

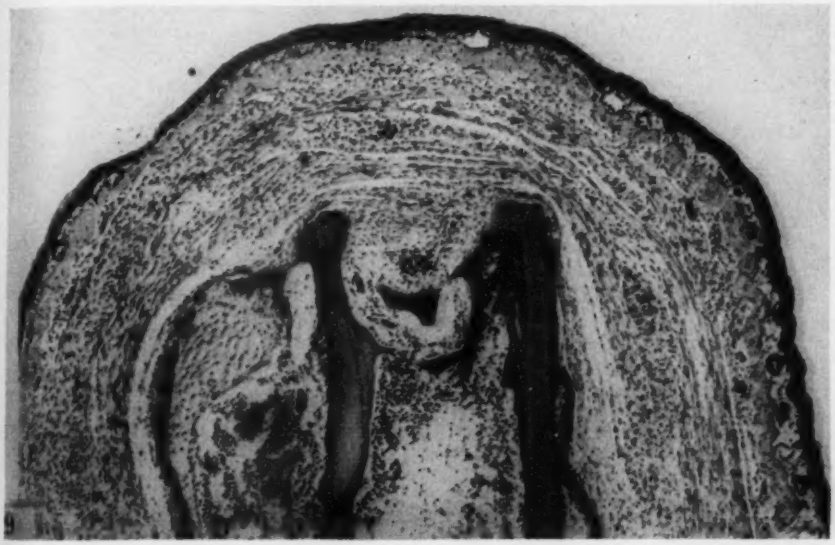

FIGURE 9. Photomicrograph of a longitudinal section from a left forelimb of a post-metamorphic *Rana clamitans* amputated through the lower arm and fixed 97 days after amputation. The skin at the amputation surface is fully reconstructed as shown by the numerous functional skin glands, a thick basal membrane; the quiescent status of the epidermis is evidenced by the normal number of cell layers. The amputated distal end of the ulna is capped by a callus, most prominent on the left side of the bone. The callus formations have become a fully integrated part of the ulna; the transformation of a large portion of the proximal portion of it into bone trabeculae is clearly visible on the left; moreover, parts of the still cartilaginous callus have acquired a periosteal bone collar and a functional periosteum as well. Distally from the callus and the bone shafts concentric layers of fibrous connective tissues intermingled with muscle fibers offer the characteristic aspect of a non-regenerating limb (60 ×).

Because of the appearance of bony differentiations at the proximal end of the callus where it fuses with the periosteum of the bony shaft of the ulna it is inferred that the growth phase of the appositional callus is terminated. This observation is also supported by the numerous sites of insertion of muscular bundles and of tendons visible on the newly formed periosteum. All the conditions of a terminated organogenesis and of a nearly completed histogenesis are fulfilled. That is to say, the frog limb after the severe amputational trauma inflicted a hundred days prior to fixation has reacquired the tissue equilibrium of a fully developed organ at rest.

Recapitulation

The foregoing observations have shown that the healing processes following amputation in a mouse digit are unique, and that they differ from what has previously been described in non-amputational wound healing processes. This statement is based upon the following considerations.

After Marchand (*op. cit.*) it has been customary to classify skin wounds in two categories: firstly, those that show a small wound with little tissue loss and in which the epidermis heals over the wound without being preceded by granulation tissue formation are said to heal by primary intention; secondly, those that involve gross defects and extreme tissue loss involving particularly the dermis, heal more slowly, are characterized by accumulation of granulation tissue before epidermal healing may commence, and these are said to heal by secondary intention.

The wound produced by amputation of a digit certainly involves extensive tissue loss in an organ with a static morphological organization, and thus one would expect the features characteristic of a secondary type of wound healing. But all our observations concur to show precocious epidermal healing: as early as three days after amputation a completely joined epithelium was shown to exist; moreover, this epithelium was in many instances separated from the cut end of the bone by only a fluid area of tissue debris, and a completely re-formed epidermis was visible before any signs of dermal proliferation and repair had occurred. These features are certainly characteristic of primary wound healing, not of healing by secondary intention.

Lindquist (*op. cit.*), in an extensive study of skin wounds on the backs of rats, concluded that it was the contraction of the tissues around and beneath the wound which was more important for closure than was epidermal migration. Although the nature of the skin and of the soft tissues covering a mouse digit is different from that found on the back of a rat, our observations support the view of Lindquist that contraction plays an important role in the healing of these amputational wounds: while during the first day the amputation surface remains large (Fig. 1), by the third day of healing the wound surface is greatly reduced in size, and the actual extent of epithelial migration is small (Fig. 4). It also appears likely that the appearance of an amorphous fluid space between the healed epidermis and the cut bone is at least in part due to rapid epidermal closure. The subsequent organization of this space into a bursa-like cavity with its own lining is probably a secondary reaction to irritation within the amputation stump.

Another feature which distinguishes our observations from previously reported investigations concerns the bone callus. The illustrations shown offer evidence that in amputational wound healing the callus differs from that found in normally healing fractures. Following amputation through the diaphysis the formation of new bone trabeculae is irregular and diffuse thus producing a somewhat shapeless callus; however, amputation through the epiphysis results in mere destruction of the bone with little new callus formation. The irregular nature of the callus is no doubt due to the absence of a bone fragment in apposition to the injured stump, undoubtedly a condition which, in fractures, aids in the induction and organization of the proliferating elements into a more regular callus.

Conclusion

The investigator of wound healing processes (surely an unsatisfactory term) cannot help but be impressed and awed with the organism's ability to respond in such a complex fashion to the stimulus of a simple amputation. The loss and destruction of tissues determined by infliction of a wound stimulates in some as yet unknown way (see the thoughtful discussion on the subject of "New Tissue Formation in an Adult Mammal" by Abercrombie, 1957) first of all the migration and proliferation of the various cells within the amputation stump; but, more particularly, it also reawakens processes characteristic of ontogenetically earlier stages that lead to organization of these new and old cells into morphogenetically distinct tissues and organs. Note, for example, the formation of the large bone callus, the heavy cap of connective tissues of the end of the bone, and the appearance of a walled-in bursa-like cavity, transitory as it may be. Every one of these reparative and morphogenic mechanisms is surely under some form of systemic control, be it "wound hormones," nerves or endocrines (Abercrombie, *op. cit.*).

While it is surely essential for the student of regenerative processes to understand the causative factors involved in this post-amputational proliferation and migration of cells, the comprehension of the reasons for their precocious and apparently "final" differentiation into equilibrated structures appears still more imperative. To one accustomed to observing the properties of regeneration in urodeles where organological equilibrium is achieved a long time after amputation, it is most important to understand why amputation of a mammalian appendage leads within such a limited time to that tissue and organ equilibrium which is the very essence of arrest of growth and development.

For these reasons we have thought it rewarding to compare post-amputational wound healing in a mammal, with the type of healing seen in the amputated limb of a post-metamorphic frog, an animal that before metamorphosis possessed the ability to regenerate amputated limbs. The comparison of the histological features of amputated limbs from two non-regenerating animals offers convincing evidence, we believe, of fundamental similarities in their patterns of wound healing. In fact, there is nothing in the histological appearance of either of these two appendages that would suggest that one of these non-regenerating limbs would be more susceptible to respond to treatments that might awaken regeneration than the other. Yet, workers of the past two decades have shown that limbs of post-metamorphic frogs can be induced to regenerate: by surgical trauma (Polejaiev, 1936), chemical trauma (Rose, 1944), by augmentation of nerve supply (Singer, 1954) and finally by altering the systemic hormonal balance (Schotté and Wilber, 1958).

In view of the success of these experiments with frogs, animals with wound healing patterns that are strikingly similar to those of mammals, it is legitimate to expect that modifications of at least some aspects of wound healing processes may also be observed in mice under the influence of some experimental devices. Some such responses in healing patterns of mouse digits have already been obtained and they will be reported in a forthcoming paper.

Summary

1. A systematic study of the early stages of amputational wound healing in mouse digits is presented. Digits were regularly amputated through the middle

phalanx and a total of 191 cases were fixed for histological study at periods ranging from six hours to three weeks after amputation.

2. The first phases of post-amputational healing, characterized by provisional closure of the wound by a blood clot and by subsequent inflammation, were found to be similar to non-amputational wounds, but differences were observed in the patterns of epidermal, dermal and bone healing.

3. Epidermal healing began six hours after amputation and was completed in most cases by three days. Dermal and sub-dermal connective tissues showed the first signs of healing not earlier than three days after amputation and it was generally completed one week after amputation. The observation that epidermal closure of the wound preceded any signs of dermal repair indicates that these amputational wounds heal by "primary intention." It was also observed that tissue contraction contributed to this type of healing.

4. Depending upon the level through which the phalanx was amputated, two types of callus formations were observed: (a) amputation through the diaphysis resulted in the formation of a large callus that was more diffuse and amorphous than those previously described for healing fractures; (b) amputation through the epiphysis resulted in very little callus formation, often concomitant with destruction of bone.

5. An unusual aspect of amputational wound healing in mouse digits was the appearance of bursa-like formations between the cut bone and the healed epithelium; at three days a fluid space formed which subsequently developed into a structurally distinct cavity with the morphological characteristics of a bursa. However, these structures were only transitory and they disappeared during the second post-amputational week.

6. Two weeks after amputation the mouse digit was found to be almost completely healed. Additional growth in the form of a heavy cap of connective tissues arranged in parallel layers enclosing the distal and lateral parts of the cut bone was an invariable feature of this healing. The rapid return to equilibrium of the tissues within the amputation site was compared to a similar type of healing observed in amputated limbs of post-metamorphic frogs.

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THE MORPHOLOGY AND LIFE-HISTORY OF THE DIGENETIC TREMATODE, ASYMPHYLODORA AMNICOLAE N. SP.; THE POSSIBLE SIGNIFICANCE OF PROGENESIS FOR THE PHYLOGENY OF THE DIGENEA

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Constitution of the Genus Asymphylodora

The genus *Asymphylodora* was erected by Looss (1899) to contain *Distoma perlatum* von Nordmann, 1832, a parasite from the intestine of *Cyprinus tinca*, as type species. Rudolphi (1809) had suggested and Looss agreed that the species is identical with *Fasciola tincae* Modeer, 1790, from the same host. In the genus *Asymphylodora*, Looss included *Distoma exspinosum* Hausmann, 1896 from the intestine of *Barbus fluviatilus* and *Distoma imitans* Mühling, 1898 from the intestine of *Abramis brama*. *Distoma ferruginosum* von Linstow, 1877 from the intestine of *Barbus fluviatilus*, was considered identical with *D. perlatum*. *Fasciola globiporum* Rudolphi, 1802 was listed as *Distoma globiporum* by Rudolphi (1809) who stated that it included *F. tincae* and several other inadequately described trematodes. Looss (1894) had given a detailed description of *D. perlatum* and although he admitted the identity of this species and *F. tincae*, he did not make the correct taxonomic revision when he erected the genus *Asymphylodora*. Lühe (1909) made the combination *Asymphylodora tincae* (Modeer, 1790); he recognized the validity of *Distoma ferruginosum*, which he included with the three species selected by Looss as members of the genus. *Distoma punctatum* Zeder, 1800 from the intestine of *Cyprinus barbus*, was listed as probably identical with *Asymphylodora ferruginosum*. All of these worms were from the intestine of cyprinid fishes of Europe.

Subsequently, members of the genus have been reported from various parts of the world. Isaichikov (1923) described specimens from cyprinid fishes of the Kuban River as *Asymphylodora tincae kubanicum*, and this form was re-described by Markewitsch (1951). Ozaki (1925) described *Asymphylodora macrostoma* from several species of fishes in Japan. Ivanitskii (1928) described *Asymphylodora dneproviana* from fishes in the Ukraine. Witenberg and Eckmann (1934) found worms in *Cyprinus carpio* in Syria which they identified as *A. tincae*. After noting the variability manifested by different individuals, they declared that all previously described species were identical and accordingly, all other specific names were reduced to synonymy with *A. tincae*. Markowski (1935) described *Asymphylodora demeli* from *Gobius minutus*, taken in the coastal waters of Poland. He

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accepted *A. macrostoma* as a valid species and asserted that the action of Witenberg and Eckmann was not justified, since it was based on evaluation of published descriptions rather than on comparison of specimens. Srivastava (1936) described *Asymphylodora indica* from the intestine of the fresh-water fish, *Ophiocephalus punctatus* in India. Yamaguti (1936) described *Asymphylodora diplorchis* from *Pseudogobio esocinus;* a species which according to Yamaguti (1958) had been described by Takeuti (1936) as *Steganoderma kamatsukae.* Yamaguti (1938) described *Asymphylodora japonica,* a species from *Cyprinus carpio* which had been identified by Nagano (1930) as *A. tincae.* In his report, Yamaguti (1938) stated that the asexual generations of this species occur in *Bulimus striatulus japonicus* and that the tailless cercariae encyst in the rediae. He emended the diagnosis of *A. macrostoma* and reported that larvae of this species encyst in the peribuccal tissue or gill arches of *Chaenogobius macrostomus, Gnathopogon elongatus caerulescens,* and *Cobitus biwae.* Serkova and Bykhovskii (1940) reported both redial and sexually mature stages of a species of *Asymphylodora* in *Bithynia tentaculata* collected near Leningrad, Russia. The worms were distinguished from previously described species by a tabular comparison of morphological features. Although the authors stated that only experimental studies under natural conditions, *i.e.,* the infection of fishes with young trematodes, will permit definite systematic disposition of the specimens, they were described provisionally as a new species, *Asymphylodora progenetica.*

Szidat (1943) considered the material of Witenberg and Eckmann to consist of two species, one of which he identified as *A. imitans* (Mühling, 1898) and the other he described as a new species, *Asymphylodora carpiae.* The latter species was regarded as identical with *A. tincae kubanicum* Isaichikov, 1923, and if this is true, *A. carpiae* is a synonym of *A. kubanicum.* Szidat distinguished between *A. tincae, A. exspinosa, A. ferruginosa, A. carpiae* and *A. demeli;* he attempted to assign species of *Asymphylodora* to particular hosts, *e.g., A. tincae* occurs apparently in a single host species, *T. tinca,* although other species are not so restricted. For the Asiatic species, *A. macrostoma* Ozaki, 1925 and *A. indica* Srivastava, 1936, Szidat erected a new genus, *Parasymphylodora,* which was suppressed by Skrjabin (1955). Szidat also erected a new genus, *Paleorchis,* for the species with two testes, *i.e., A. diplorchis* Yamaguti, 1936 and two species which he described as new, *viz., P. incognitus* from the intestine of *Leuciscus rutilus* and *P. unicus* from the intestine of *Blicca björkna.* The three genera, *Asymphylodora, Parasymphylodora* and *Paleorchis* were included in a new subfamily, Asymphylodorinae. This subfamily was assigned to the family Lecithodendriidae Odhner, 1910, together with the subfamilies Lecithodendriinae Looss, 1902; Pleurogenetinae Looss, 1899; Haploporinae Looss, 1902; Monorchinae Odhner, 1911; Proctotreminae Odhner, 1911; and Zoogoninae Odhner, 1911. Szidat (1943) stated (p. 70), "Unter Hinweis auf schon früher (1926) erhobene Befunde wird dargelegt, dass die genannten Trematodengruppen lediglich als Unterfamilien der grossen Trematodenfamilie Lecithodendriidae aufzufassen sind, womit ein neuer Beweis für die von dem Autor vertretene Auffassung einer *Parallelentwicklung der Trematoden mit ihren Wirten im Laufe der Phylogenie* erbracht wird und die von W. EICHLER aufgestellten Korrelationsregeln in der Stammesentwicklung von Wirten und Parasiten, die sog. FAHRENHOLZsche und die SZIDATsche Regel erneut bestätigt werden konnten."

Other species, subsequently described as new, include *Asymphylodora atherinopsidis* from the "jack smelt" *Atherinopsis californiensis* taken at Stinson's Beach, California by Annereaux (1947); *Asymphylodora markewitschi* from *Carassius carassius* taken in Russia by Kulakowskaja (1947); *Asymphylodora pontica* from *Neogobius melanostomus* taken in Odessa Bay by Tschernyschenko (1949); *Asymphylodora kedarai* from *Punctius sophora* taken in India by Srivastava (1951); *Asymphylodora macracetabulum* from *Misgurnus anguillicaudatus* taken in Russia by Belous (1954); and *Asymphylodora dollfusi*, a progenetic metacercaria from *Bithynia leachi* taken in the north of France by Biguet, Deblock and Capron (1956). It should be noted that Markewitsch (1951) predicated the identity of *A. dneproviana* and *A. imitans* (Mühling, 1898) and Skrjabin (1955; p. 408) confirmed the opinion. Certain of the species of *Asymphylodora* are described only briefly and others, *e.g., A. atherinopsidis*, which was described from a single worm, are based on a small number of specimens. Which of the twenty named species are actually valid remains to be determined.

Life History and Larval Stages

The life-cycle of members of the genus *Asymphylodora* has been the subject of much controversy. It presents many interesting features and has been under investigation for more than a century. Von Baer (1827) described *Cercaria paludinae impurae* and the species described by de Filippi (1854) from the same host under the name *Distoma paludinae impurae* may be identical with it. It is impossible, however, to make positive determinations on the basis of those early accounts. In the species described as *D. paludinae impurae*, de Filippi reported the finding of a large number of rediae, which produced the distomes directly. These rediae had very short, dilated intestines and no lateral processes of the body wall. The young rediae were motile and moved in a lumbricid manner, while the older ones were inert and contained active distomes, about 0.40 mm. in length, which strongly resembled *Distoma luteum* von Baer, 1826 from *Paludina vivipara*. In this paper, de Filippi reported daughter rediae in a mother generation. He predicated that the production of distomes in a redia is not to be interpreted as an example of simplified development. He stated that these distomes differ from cercariae only in the absence of a tail; he observed cystogenous cells under the body wall, but noted that encystment occurs only outside of the body of the *Paludina*. In a footnote, he described distomes found in *Paludina vivipara* from the Lac de Varése, which measured up to 2 mm. in length and in which the sexual organs were present as rudiments. The worms had no fixed location, but moved about and were found on the mantle. De Filippi expressed the belief that they had come from some other animal and had merely taken refuge in *P. vivipara*. He stated that they were quite distinct from the cercariae which are normal parasites of these mollusks. This large species may be identical with the one from *P. vivipara* described by Swammerdam in the Biblia Naturae (Boerhaave edit. 1752, p. 75, Table IX, Figs. 7, 8) and by von Baer (1826) as *Distoma luteum*.

In a second mémoire, de Filippi (1855) described other distomes from mollusks. These observations demonstrated that tailless cercariae occur in the life-cycles of very different trematodes; that they may be produced in either sporocysts or rediae, and that they may be found in snails without the antecedent generation in

which they originated. The absence of a tail, then, is no evidence of genetic relationship.

In a third mémoire, de Filippi (1857) reported that *Distoma paludinae impurae* occurs in two forms which he designated ***armatum*** and ***inerme***, respectively, the latter of which was recognized as the larval stage of ***Distoma perlatum*** von Nordmann. As noted, Looss (1894) gave a detailed description of *D. perlatum*. He identified the two forms *Distoma paludinae impurae armatum* and *Distoma paludinae impurae inerme* as developmental stages of *D. perlatum* and reported that the cercariae develop in *Bithynia tentaculata*. These two larvae had been transferred to *Cercariaeum* by Diesing (1858) and Szidat (1943) suggested that Looss had found only *D. paludinae impurae armatum*. According to Looss, the cercariae may leave the snail in which they were produced and they may encyst either in the same snail or in others in which no redial stages are present. According to Looss, Wagener (1857) had observed and "hübsch gebildet" this species, but had assigned it erroneously to *Cercaria lymnaei auricularis* de Filippi, 1854. Lühe (1909) accepted the statement of Looss that *Cercariaeum paludinae impurae* from *Bithynia tentaculata* is a larval stage of *Asymphylodora tincae*. He wrote (p. 93) "Die Cercarie, *Cercariaeum paludinae impurae* (Fil.), (schwanzlos, 0.4 mm. lang) entwickelt sich in *Bithynia tentaculata* (L.) in Redien, deren dicker, sackförmiger Darm ungefähr bis zur Grenze der beiden ersten Drittel der Körperlänge reicht (vgl. Fig. 186). Ein Hilfswirt dürfte bei der Schwanzlosigkeit der Cercarie fehlen." In this work Lühe erected the group, Cercariae, with two genera, *Cercariaeum* and *Leucochloridium*, to contain tailless larvae. Members of *Cercariaeum* develop in rediae or unbranched sporocysts and do not encyst. In it he included *C. limnaei obscuri* Ercolani, 1881 and *C. planorbis carinati* de Filippi, 1857 which develop in rediae, together with *C. lymnaei auricularis* de Filippi, 1854 and *C. ancyli lacustris* Diesing, 1855 which develop in sporocysts. Members of *Leucochloridium* develop in branched sporocysts. Sewell (1922) divided the Cercariaea into three sections; *Leucochloridium* was retained but *Cercariaeum* was split into two parts, the "Mutabile Group" which develop in rediae and was named for *C. mutabile* Cort, 1918 and the "Helicis Group" which develop in unbranched sporocysts and was named for *C. helicis* Meckel, 1846. Meanwhile, Fuhrmann found redial and cercarial stages in *Limnea auricularis* var. *ampla* taken in the region of Neuchâtel, Switzerland which he (1916) described as *Cercariaeum squamosum*. The rediae were 2.2 to 3.5 mm. long and the cercariae 0.36 mm. long. The cercariae were covered, except for the ventral area between the suckers, with cuticular scales which were similar to those of species of *Asymphylodora*. Fuhrmann postulated that *C. squamosum*, rather than *C. paludinae impurae* as stated by Lühe (1909), is a larval stage of *A. tincae*.

Dubois (1929) described the redial and cercarial stages of *Cercariaeum squamosum* and reported the species from *Lymnaea limosa, Lymnaea stagnalis* and *Planorbis carinatus*. The rediae were described as simple, saccate structures and Dubois noted such variability in dimensions of body and suckers of cercariae from different individuals of the same species, that he regarded the tailless larvae from all the above named snails as specifically identical. In the Cercariaea, he recognized five groups:

1. Groupe Mutabile. These larvae develop in rediae; they have two testes; the excretory vesicle is tubular and flexible; the flame-cell formula is 2 [(4 + 4 + 4 + 4) + (4 + 4 + 4 + 4)].

2. Groupe Helveticum. These larvae develop in rediae; they have two testes; the excretory vesicle is tubular and flexible; the flame-cell formula is 2 [(3 + 3 + 3) + (3 + 3 + 3)].

3. Groupe Squamosum. These larvae develop in rediae; they have a single testis; the excretory vesicle is small, pyriform or rounded; the flame-cell formula is 2 [(4 + 4 + 4 + 4) + (4 + 4 + 4 + 4)].

4. Groupe Helicis. These larvae develop in sporocysts; number of testes and form of excretory system uncertain.

5. Groupe Leucochloridium. These larvae develop in sporocysts; number of testes and form of excretory system uncertain.

Dubois' observations upheld the morphological agreement, noted by Fuhrmann, between *C. squamosum* and *A. tincae* and accordingly he recognized the identity of the two forms. In the Groupe Squamosum he included *C. paludinae impurae inerme* together with *C. planorbis carinati* and *C. limnaei obscuri*. According to Dubois, *C. paludinae impurae armatum* has a small stylet, two testes, a tubular excretory vesicle, and since it has a flame-cell formula of 2 [(3 + 3 + 3) + (3 + 3 + 3)], it was included in the Groupe Helveticum.

Wesenberg-Lund (1934) described tailless larvae from *Bithynia tentaculata* which he identified as *C. paludinae impurae*. In general morphology these larvae agreed with those from the same host which were described by Dubois (1929) as *Cercariaeum helveticum* I and which were included together with *C. paludinae impurae armatum* to form the Groupe Helveticum. Wesenberg-Lund did not observe a stylet, but otherwise the two forms were very similar. Redial stages and encysted metacercariae were found in the same snail. The cyst walls were thin and membranous. Wesenberg-Lund confirmed the observations of Wunder (1924) that the larvae emerge from the spiracle of the infected snail, pass over the head and proceed along the tentacles, which may appear fluffy in their entire length, with as many as fifty worms on each, or the larvae may accumulate at the tips. The cercariae are always attached by their ventral suckers, with the anterior and posterior portions elevated. If the tentacles come in contact with other snails, the larvae may transfer to the new hosts where they later encyst, usually near the heart or rectum. The author agreed with Dubois that the *inerme* and *armatum* forms of de Filippi are distinct species, but no attempt was made to relate either of them to an adult form. Wesenberg-Lund identified other tailless larvae from *Lymnaea auricularia* as *Cercariaeum lymnaei auricularis* de Filippi, 1854 and his description of these specimens agrees closely with that of *C. squamosum* from the same host as given by Fuhrmann (1916) and Dubois (1929). The two forms are obviously related, but their relation to *A. tincae* is undetermined.

As noted, Serkova and Bykhovskii (1940) described *Asymphylodora progenetica* from the liver and other organs of *Bithynia tentaculata* in Russia. They reported that more than 50 per cent of the mollusks were infected, each with one to seven worms; about 60 per cent of the worms were juveniles, the others sexually mature with eggs in the uterus. None of these worms was encysted. In addition to the juvenile and mature worms, some 15 to 20 per cent of the snails harbored

rediae which were presumed to belong to the same species. Each redia contained from one to eight tailless cercariae. Three sets of experiments were performed. In the first, uninfected specimens of *B. tentaculata* were placed in aquaria with infected ones. At the end of one month, both juvenile and gravid worms were found in the previously uninfected snails. The worms were always free; none was encysted. No rediae were found in the previously uninfected individuals. There was no statement concerning the number of snails used in the experiment nor of the criteria used to determine whether or not the snails were infected. In a second experiment, fifteen uninfected specimens of *B. tentaculata* were placed in each of three aquaria and eggs of *A. progenetica* were added. At the end of twenty days the following results were obtained: in Aquarium I, there was one snail which contained an embryonic mass, presumed to be of *A. progenetica,* and the others were not infected; in Aquarium II, one snail contained eight mature specimens of *A. progenetica* with eggs in their uteri and four dead worms, while the other snails were not infected; in Aquarium III, none of the snails was infected. From these results, the authors concluded that the life-cycle of *A. progenetica* can be completed in *B. tentaculata,* and that the development from eggs to mature adults can take place in twenty days. In the third experiment, pieces of *B. tentaculata* were fed to cyprinid minnows, *Rutilus rutilus* and *Carassius carassius.* In *R. rutilis,* the worms persisted for six days but were absent after fifteen days; in *C. carassius,* the worms were present after twenty-four hours, but not at the end of three days. The experiments reported by Serkova and Bykhovskii are not at all convincing. In the absence of information concerning controls, the results can not be evaluated properly. However, the belief that *B. tentaculata* harbors a single species, and that the rediae and cercariae were larval stages of the species whose juvenile and sexually mature stages were described as *A. progenetica,* was not adequately supported. The results of the attempt to infect *B. tentaculata* discredit the stated conclusions. The failure to obtain rediae suggests the absence of experimental infection. The finding of an embryonic mass in one snail is equivocal, and the snail with eight gravid worms was certainly infected at the time of exposure.

Szidat (1943) confirmed earlier observations of Dubois (1929) and Wesenberg-Lund (1934). He reported that the adult stage of *C. paludinae impurae armatum* occurs in *Leuciscus rutilus* and for it he erected a new genus, *Paleorchis.* He noted that the first three groups of Dubois are closely related, whereas the fourth and fifth groups, which develop in terrestrial snails, are members of a different family, Harmostomidae (= Brachylaemidae). Szidat declared that the larvae of *Asymphylodora* certainly belong in the Squamosum group of Dubois. He recognized *C. limnaei auricularis* de Filippi, 1854 and *C. squamosum* Fuhrmann, 1916 as distinct species, and the former was identified as the larva of *A. tincae.* Provisionally, the latter species was referred to *A. imitans* (Mühling, 1898). Szidat considered the probability that *C. planorbis carinati* de Filippi, 1857; *C. limnaei obscuri* Ercolani, 1881; and *C. squamosum* Fuhrmann, 1916 are identical with *C. paludinae impurae inerme* de Filippi, 1854.

The five larval groups of Dubois have now been correlated with their adult stages. Wallace (1941) reported that *C. mutabile* Cort, 1918 is the larva of a species of *Triganodistomum* Simer, 1929. The Helveticum group contains the lar-

vae with stylets, two testes, which according to Wunder (1924) leave their primary hosts and encyst in secondary hosts. They were identified by Szidat (1943) as developmental stages of species of *Paleorchis*. Members of the Squamosum group are larvae of *Asymphylodora*. Thomas (1959) transferred *Triganodistomum* to the Monorchiidae and thus, members of the three groups belong in the family Monorchiidae or in the subfamily Monorchiinae if the proposal of Szidat is accepted and the monorchids are regarded as a subfamily in the expanded family Lecithodendriidae.

The identity of corresponding stages of species in the genus *Asymphylodora* is yet undetermined. Deblock, Capron and Biguet (1957) reported specimens of *Asymphylodora* from the intestine of *T. tinca* taken in the north of France. These worms differed from descriptions of *A. tincae* as given by earlier authors, including Szidat (1943) who associated *A. tincae* exclusively with *A. tinca*. The worms described by the French authors were differentiated from previous descriptions of *A. tincae* on the basis of three features, *viz.*, the presence of a short common sperm duct before the seminal vesicle, the extension of ventrolateral pores to converge and meet behind the acetabulum, and the absence of spines on the ventral area between the suckers. The worms were designated as a new variety, *Asymphylodora tincae mediaglabra*. The question was raised if the larval form of the classically described form of *A. tincae* is not *C. paludinae impurae inerme* as postulated originally by de Filippi (1857).

Experiments

Stunkard (1955) reported the finding in the summer of 1954 of both juvenile and sexually mature trematodes in the fresh-water pectinibranchiate snail, *Amnicola limosa*, collected from ponds in the vicinity of Woods Hole, Massachusetts. About one-fourth of the fully grown snails were infected. From one to nine unencysted worms, identified as members of the genus *Asymphylodora*, were found in an infected snail. As a rule, when several specimens were present only one or two contained eggs, but as many as five gravid worms were found in a single snail. The presence of worms of different sizes and degrees of development, from very young to gravid specimens, indicated successive repeated infections. When removed to a glass slide, juvenile specimens frequently encysted but encystment of sexually mature worms was not observed. The cyst walls were thin and membranous. No sporocyst or redial stages were found at the time and it appeared that the asexual generations of the worms occur in some species other than *A. limosa*.

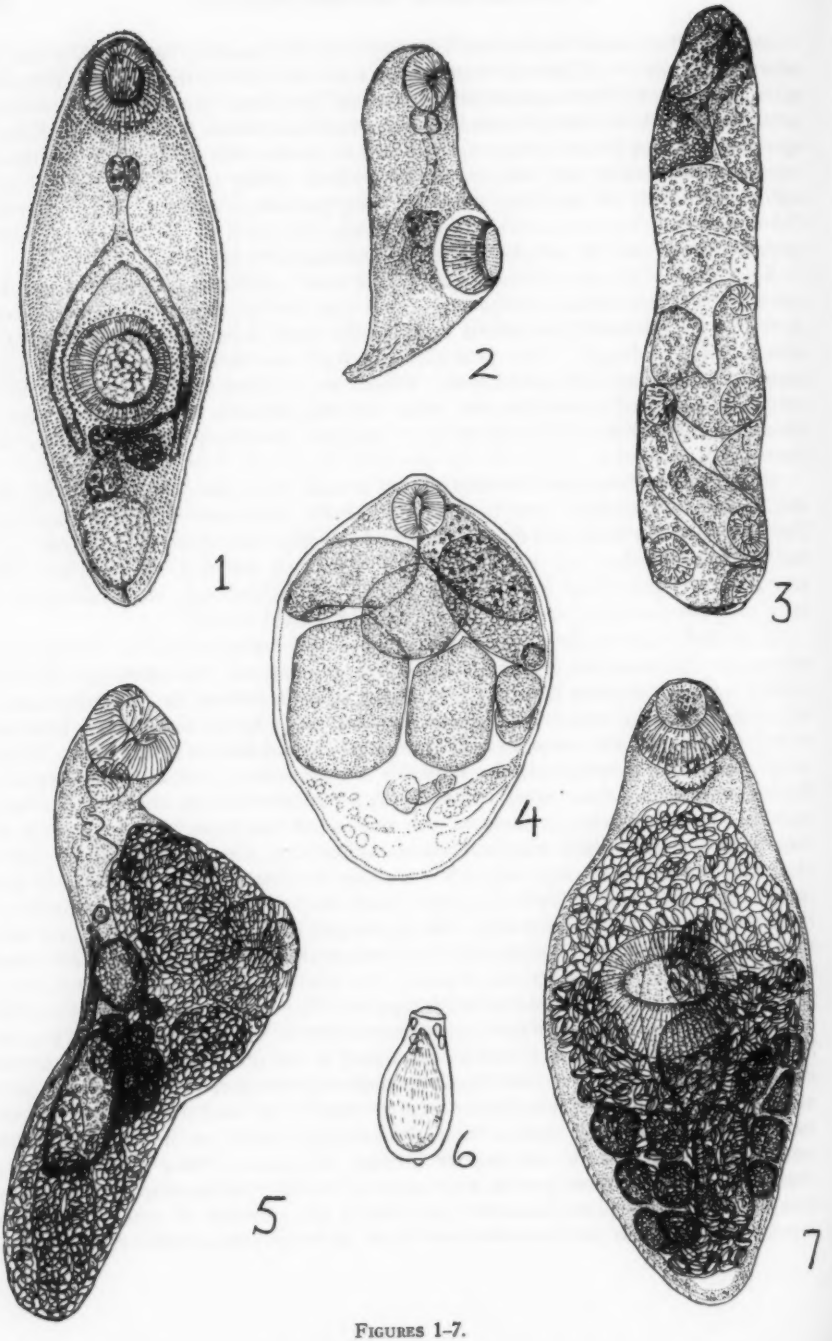
The worms were found, but only rarely and in small numbers, in the intestines of fishes from the same ponds. They were taken from yellow perch (*Perca flavescens*), fresh-water killifish (*Fundulus diaphanus*), small-mouth bass (*Micropterus dolomieu*) and bluegill sunfish (*Lepomis macrochirus*). Whether the fishes obtained the worms by eating snails or by eating small fishes which had eaten snails is unknown. Trematodes from ingested fishes may persist for some time in the intestines of predatory animals and parasites of the prey may easily be mistaken for parasites of the predator. The eggs of the parasite contained miracidia and those in worms from snails were identical in appearance with those in worms taken from the intestine of fishes. The ponds had been stocked at various times by the Division of Fisheries and Game of the State of Massachusetts.

Subsequently, redial and cercarial stages of this parasite have been found in natural infections of *A. limosa;* from one to three per cent of the snails in different collections. In addition, sporocysts and rediae have been recovered from experimentally infected, laboratory-reared snails. *Amnicola limosa* is a dioecious species; eggs are laid singly and young specimens are present in numbers in laboratory cultures. The snails are temperamental feeders, living principally on diatoms, and it is difficult to maintain them for long periods, especially when infected. Cultures of diatoms were provided by Dr. Luigi Provasoli of the Haskins Laboratories in New York, for which grateful acknowledgment is made.

Eggs passed by worms taken from snails were maintained in pond water for one to six days to insure embryonation and then fed to young, laboratory-reared *A. limosa*. Each snail was placed in a stender dish, 5 cm. in diameter, together with a number of eggs. The next day the snail was removed to a larger container for more favorable conditions. Dissection of these snails, beginning after one week, disclosed sporocysts and rediae indistinguishable from those of natural infections. Whether or not there is a daughter sporocyst generation was not determined.

Cercariae removed from the tentacles of a snail were placed in a small stender dish, 3 cm. in diameter, with a small uninfected, laboratory-raised *A. amnicola*. The following day they had disappeared and the snail was dissected. About one-half of the cercariae had encysted. This finding is difficult to interpret since very few cysts have been found in snails from ponds, although often one-third or more of them contained unencysted juvenile or gravid worms.

If a snail harbors the asexual generations, the tailless cercariae emerge and migrate to the tentacles where they cling in the manner described by Wunder (1924) and Wesenberg-Lund (1934). The worms adhere by the acetabulum, with the ends of the body extended and elevated. The larvae may remain attached at any location on the tentacles and sometimes accumulate near the tips. When many cercariae are attached, the tentacles may manifest a feathery appearance. If such an infected snail makes contact with an uninfected one, the cercariae may transfer to the tentacles or body of the uninfected specimen. In the new host, they often encyst in thin membranous envelopes that are not reinforced with a cyst wall deposited by the host. If cercariae are removed from tentacles and placed in a small stender with the same snail, they soon regain their position on the tentacles. If placed in a dish with an uninfected snail, they attach and may migrate to the tentacles, but they do not remain there; instead, they move about and in most instances enter the tissues. In snails that do not harbor asexual generations, the worms may be recently encysted, they may be unencysted juveniles of older infections, or unencysted gravid specimens. Such worms often contain hundreds of eggs and those in the terminal part of the uterus contain fully formed miracidia. No rediae have been found in snails that harbored juvenile or sexually mature worms, and no juvenile or mature worms in snails that harbored the asexual generations. It appears that the cercariae leave an infected snail and enter only snails that do not harbor asexual infections. Since several worms, often two to four of them gravid, may occur in a snail, the presence of one worm does not confer complete immunity and inhibit the entrance of others. Juvenile worms may encyst or remain unencysted since such specimens retain their cystoge-



FIGURES 1–7.

nous glands for some time and can encyst if removed from their hosts. The worms grow in their cysts since they increase somewhat in size, but major growth and maturity take place in unencysted specimens. It appears if the metacercariae encyst, that later they emerge because all older and gravid worms were free in the tissues of the snail.

Descriptions

The adult (Figs. 5, 7)

The worms are fusiform, wider near the middle and narrower at the ends. The preacetabular portion of the body is almost cylindrical and since the acetabulum is large and often protruding, fixed and stained specimens are frequently oriented on the side (Figs. 2, 5), with the dorsoventral measurement greater than the width. In cercarial and juvenile specimens the acetabulum is in the posterior half of the body but with sexual maturity and the accumulation of eggs in the uterus, the acetabulum is shifted relatively farther forward and may come to lie in the anterior half of the body. The worms continue to grow after sexual maturity is attained. Specimens from fishes as a rule are somewhat larger than those from snails. Gravid worms from snails when fixed, stained and mounted measure from 0.28 to 0.58 mm. in length and 0.14 to 0.24 mm. in width; corresponding measurements of worms from fishes are 0.40 to 0.71 mm. and 0.18 to 0.25 mm. The acetabulum measures 0.09 to 0.13 mm. in diameter.

The anterior end of the body in juvenile specimens bears a number of protrusible papillae, each provided with a seta, and these papillae are sometimes visible in gravid specimens. Other papillae are present at the rim of the acetabulum. The cuticula has a characteristic spination. Over much of the anterior half of the body the spines are slender, sharp, distinctly separated and 0.006 to 0.008 mm. long. On the ventral side, the median area between the suckers lacks spines and on the dorsal side in the corresponding area the spines are reduced in size and number. On the posterior part of the body the spines are broader, more scale-like in appearance and less numerous. Around the openings of the suckers there are six to seven rows of very small spines. They are closely set, triangular in shape and manifest a serrate appearance. From these areas, the spines gradually change in size and shape to conform with the aciculate and scale-like types on the adjacent areas. The parenchyma of the body, especially in the anterior portion, contains a large number of glandular cells; the ducts of many of them lead forward to open near the anterior end of the body. Other glandular cells open

The figures, except that of the egg, were made from fixed and stained specimens.

Figure 1. Metacercaria, removed from cyst; much flattened for study of the excretory system; ventral view, 0.43 mm. long.

Figure 2. Cercaria, removed from a redia; lateral view; 0.19 mm. long.

Figure 3. Redia; natural infection; a large specimen; 0.9 mm. long.

Figure 4. Redia; one of four, from experimental infection of a laboratory-raised snail; 0.19 mm. long.

Figure 5. Adult; lateral view; one of the largest specimens; from *Perca flavescens;* 0.70 mm. long.

Figure 6. Egg, extruded from a gravid worm after removal from a snail, examined in water; 0.027 mm. long.

Figure 7. Adult; ventral view; somewhat compressed; from *Amnicola limosa;* 0.49 mm. long.

on the ventral side, especially in the area between the suckers, but rows of pores as reported by Looss (1894) and by Deblock, Capron and Biguet (1957) were not apparent. Such a linear arrangement would probably be produced only when that portion of the body is extended, otherwise the alignment would be disturbed.

The oral sucker is 0.06 to 0.095 mm. in diameter. The mouth is subterminal; there is a short prepharynx and the pharynx measures 0.036 to 0.044 mm. in diameter. The esophagus is long, the length dependent on the degree of extension of the preacetabular portion of the body, and the bifurcation of the digestive tract is immediately in front of the acetabulum. The ceca extend posteriad on the dorsal side of the body and end blindly at the level of the posterior part of the testis. The worms in the snails feed on tissues, as the digestive ceca contained cells, nuclei and cellular debris that could have come only from the molluscan host.

The excretory pore is terminal; the vesicle is small, lined with high epithelial cells. The collecting ducts pass forward to the level of the pharynx where they turn backward, and shortly anterior to the acetabulum they divide into anterior and posterior branches. Each branch has four groups of four flame-cells and the formula is $2\ [(4+4+4+4)+(4+4+4+4)]$, which is characteristic for members of the Squamosum group of cercariae. These observations were made on juvenile specimens (Fig. 1) since in gravid worms the development of the reproductive organs and the accumulation of eggs in the uterus make it impossible to work out the flame-cell pattern.

There is a single testis although the presence of two sperm ducts indicates its dual nature. It is median, located near the posterior end of juvenile and young specimens. As the body becomes congested with eggs in the uterine coils, the testis is shifted more dorsad and forward; indeed the post-testicular region may be as long as the testis. The testis is oval, 0.08 to 0.13 mm. in length and 0.06 to 0.10 mm. in width. Two sperm ducts pass forward, one from each side of the testis. In certain specimens they unite immediately before the cirrus sac and in others they unite to form a short duct which leads to the cirrus sac. Within the sac, the duct expands to form a twisted or coiled seminal vesicle which may appear bipartite or tripartite. The seminal vesicle is succeeded by a short prostatic region and then the ejaculatory duct, lined with spines, leads to the common genital pore. The prostatic cells occupy most of the space in the cirrus sac in front of the seminal vesicle. The ejaculatory duct may be everted to form the protruded cirrus. The cirrus sac extends in a curved course from the level of the anterior end of the ovary to the genital pore, situated on the left side at the acetabular level.

The ovary is dextral, immediately anterior to the testis, or the two may overlap. It is oval, somewhat smaller than the testis, and measures 0.06 to 0.11 mm. in length and 0.05 to 0.09 mm. in width. The oviduct arises from a ventral prominence near the posterior end of the ovary and makes a median posterior bend, after which it expands into a small seminal receptacle from which Laurer's canal extends to the dorsal surface of the body; it then receives the common vitelline duct and expands to form the ootype, enclosed in the cells of Mehlis' gland. The initial part of the uterus contains spermatozoa. From the ootype, in young specimens, the uterus passes forward to the level of the anterior end of the acetabulum,

then back on the right side to the posterior end of the body and forward on the left side to join the metraterm. But in older, fully gravid individuals, the anterior loop extends forward to the pharynx, coils about in the preacetabular region, filling much of the body between the suckers, and the posterior loop also winds about filling and expanding this portion of the body. In general, the uterine coils lie below the gonads and vitellaria and their course can be followed by the color of the eggs which are light in the initial part of the uterus and become darker as they proceed. The metraterm is a thicker-walled, terminal part of the uterus, about one-third as long as the cirrus sac, and the lumen is lined with cuticula which bears spines, similar to those which line the ejaculatory duct. There are nine vitelline follicles on each side of the body; typically there are five in a median, longitudinal row and four in a lateral row but contraction of the body muscles and pressure from other organs may disturb this arrangement. The follicles measure 0.033 to 0.043 mm. in diameter and are situated in the region between the acetabulum and the posterior end of the testis. Large collecting ducts from the follicles of each side pass mediad on the dorsal side of the body to form a vitelline receptacle, from which the common duct leads anteriad and ventrad to join the oviduct. The eggs (Fig. 6) are ovate, slightly narrower at the opercular end and somewhat flattened on one side. There is a thickened projection or knob asymmetrically situated near the antopercular end of the egg. The young eggs are almost colorless and the cells within stain readily; mature eggs have thick, yellow shells and contain a miracidium. The eggs measure 0.025 to 0.029 mm. in length and 0.013 to 0.016 mm. in width.

The sporocyst

No sporocysts of natural infection were found. In a snail exposed on July 3 and sacrificed on July 12, there was a sporocyst which measured 0.07 by 0.52 mm. It contained only germinal cells. Another sporocyst found on July 25 in a snail exposed on July 10 was slightly larger and in addition to germinal cells it contained three small germinal masses, embryos of the next generation.

The redia (Figs. 3, 4)

The rediae are simple sausage-shaped structures, without collar or feet, typically cylindrical although often attenuated at one or both ends. The body wall contains both circular and longitudinal muscles, so the rediae are slender when elongated and plump when retracted. In size they measure up to 1.00 mm. in length and 0.2 mm. in width, although most of them are only about one-half this size. There is a birth-pore situated near the pharynx. The pharynx has a diameter of 0.035 to 0.040 mm.; the gut has a short neck-like portion and the intestine is saccate. In young, small rediae it may extend one-third of the length of the body but it does not increase appreciably in size, and in older, mature specimens it occupies only a small fraction of the body cavity. Figure 4 shows one of four rediae found in a laboratory-raised snail exposed on July 14, 1955, and dissected on the following September 7th. The rediae were all approximately the same size. The one shown in the figure measures 0.19 by 0.13 mm. and the pharynx is 0.030 mm. in diameter.

The cercaria (*Fig. 2*)

The cercariae grow to large size in the rediae. Specimens removed from rediae or taken from tentacles of snails measure from 0.16 to 0.32 mm. in length and 0.06 to 0.12 mm. in width. The oral sucker is 0.05 to 0.06 mm.; the acetabulum 0.06 to 0.07 mm.; and the pharynx 0.033 to 0.035 mm. in diameter. The reproductive organs are represented by a lobed mass of germinal cells situated dorsal and perhaps slightly posterior to the acetabulum. The excretory system was not fully worked out in cercariae, but apparently it is completed in the cercarial stage and so far as determined, is identical with that found in juvenile worms.

The metacercaria (*Fig. 1*)

Cercariae from the tentacles of a snail were placed in a small stender dish with a small, laboratory-reared *A. limosa*. The following day they had disappeared and the snail was dissected. About one-half of the cercariae had encysted. The cysts measured 0.19 to 0.22 mm. in diameter; they were very thin-walled and ruptured easily under pressure of a coverglass. A few encysted specimens have been found in naturally infected snails, but almost all the metacercariae from natural infections were unencysted. On a few occasions, juveniles removed from naturally infected *A. limosa* encysted when removed to a slide for observation. The specimen shown in Figure 1 was removed from a cyst; it is hardly more than a cercaria since it had been removed from the tentacle of a snail the day before. It was very much flattened to study the excretory system. The unencysted metacercariae feed and grow; some of the juveniles were as large as worms with eggs in the uterus. Large numbers of dead, gravid worms have been found in naturally infected snails.

Except for the single specimen from the marine silverside, *Atherinopsis californiensis*, described by Annereaux (1947) as *Asymphylodora atherinopsidis* n. sp., the genus *Asymphylodora* has not been reported previously from North America. Mature specimens from *A. limosa* are smaller than those of either *A. progenetica* or *A. dollfusi*. The suckers are smaller, but the gonads and eggs are larger. There are differences also in the number of vitelline follicles. Description of the several stages of the species found in *A. limosa* taken from ponds in the Woods Hole region indicates that the specimens can not be assigned to any known species, and they are described as a new species, *Asymphylodora amnicolae*.

Discussion

In the paper describing *Distoma paludinae impurae*, de Filippi (1854) made the first distinction between sporocysts and rediae. He noted that *Redia gracilis*, described by him (1837), was a developmental stage of an amphistome and that similar stages occur in other species. Accordingly, he declared that the term, Redia, is not a generic name but like Cercaria O. F. Müller, 1773, is merely a stage in the life-cycle of certain trematodes. He characterized sporocysts as simple, membranous sacs without internal organization, whereas rediae are provided with a mouth, muscular pharynx, and intestine. In certain species, the body of the redia may form temporary lateral processes. It was common knowledge that cercariae could be produced in either sporocysts or rediae and de Filippi reported

daughter rediae in a mother generation. The term *"méta-cercaire"* was proposed by Dollfus (1913) to designate the immediate postcercarial stage, whether encysted or not, although in later publications he wrote the name as a single word, métacercaire. The designation of this stage is a contribution which facilitates description and discussion of life-cycles among digenetic trematodes.

Sexual maturity and production of eggs by metacercariae had been known since the report by von Siebold (1835) of eggs emerging from metacercariae found in *Astacus astacus*. For sexual maturity of animals which had not yet attained adult condition, Giard (1887) proposed the term "progenèse" and Dollfus (1924) applied the designation to gravid metacercariae of digenetic trematodes. Sinitsin (1905) reported sexually mature specimens of *Pleurogenes medians* encysted in aquatic larvae of *Agrion* sp. taken in the vicinity of Warsaw. Joyeux (1923) found gravid specimens of *Ratzia parva* in cysts from *Rana esculenta* in North Africa and obtained swimming miracidia from the eggs. Mathias (1924) obtained adults of *Pleurogenes medians* in *Rana temporaria* and *Hyla arborea* after feeding encysted metacercariae from *Gammarus pulex*. Many of the metacercariae contained eggs.

Dollfus (1927) described progenetic metacercariae of *Dinurus tornatus* (Rudolphi, 1819) and wrote (p. 55), "La découverte de cette forme permet cependant de se poser une fois de plus la question de savoir s'il peut exister, chez les trématodes digénétiques, des cycles abrégés par suppression de l'hôte définitif; l'on n'a pas, toutefois, démontré que les oeufs formés par les métacercaires progénétiques soient susceptibles de donner des miracidia infectants pour le mollusque premier hôte. Il semble que l'expérience seule pourra fournir la solution de ce problème si intéressant pour la biologie générale." Fuhrmann (1928) reported progenetic metacercariae of *Ratzia parva* in cysts from the muscles of *Rana temporaria*, and Dollfus (1929) again raised the question, "Existe-t-il des cycles évolutifs abrégés chez les trématodes digénétiques? Le cas de *Ratzia parva* (Stossich, 1904)." He stated (p. 203), "La métacercaire est progénétique, elle donne des oeufs contenant un miracidium. L'on n'a pas encore pu établir si ce miracidium renferme des éléments germinaux et est infestant pour le mollusque premier hôte, mais le seul fait que ce miracidium existe et nage activement dans l'eau après éclosion de l'oeuf permet de se demander s'il n'existerait pas, chez *Ratzia* outre un cycle évolutif complet comprenant un adult vrai dans l'hôte définitif, un cycle abrégé dans lequel la métacercaire jouerait le rôle d'adult et le deuxieme hôte intermédiare le rôle d'hôte définitif." Reproduction in progenetic metacercariae of *R. parva* was discussed by Joyeux, Noyer and Baer (1930). Dollfus (1932) described progenetic metacercariae from *Planorbis planorbis* and reported (p. 412), "Nous signalons le premier cas connu d'une métacercaire progénétique chez un mollusque d'eau douce. Le même planorbe héberge des sporocysts, les cercaires, les métacercaires enkystées et les métacercaires libres, vraisemblablement sorties de leur kyste." Since the larvae attained sexual maturity in their cysts and contained both spermatozoa and developing eggs, Dollfus admitted the possibility of self-fertilization.

Alice Buttner (1950a) stated (p. 21), "Les métacercaires progénétiques sont surtout fréquentes chez arthropodes (insectes, crustacés), ainsi que chez les poissons et les batraciens; elles ont été rencontrées également quelquefois chez des mol-

lusques." She reported for *Plagiorchis brumpti* the first experimental demonstration of an abbreviated life cycle in a digenetic trematode and predicated (p. 25), "La progénèse est, chez les trématodes, un phénomène tantôt constant, tantôt accidentel; dans ce dernier cas, les métacercaires non progénétiques restent susceptibles, comme les progénétiques, de devenir adultes chez un hôte définitif." In a comprehensive study of progenesis among digenetic trematodes, Mlle. Buttner (1950b, 1951) reviewed previous accounts, listed progenetic species in more than a score of genera and a dozen different families, and reported experiments showing constant, obligatory progenesis in *Paralepoderma brumpti* and *Ratzia joyeuxi* and inconstant, facultative progenesis in *Pleurogenes medians*. Other observations were made on identified and unidentified progenetic cercariae and metacercariae.

Biguet, Deblock and Capron (1956) described progenetic metacercariae from *Bithynia leachi* collected in the north of France. Dissection of these mollusks revealed an infection rate of more than 30 per cent, with one to twelve unencysted specimens in each host. The worms manifested varying degrees of maturity and about 10 per cent of the infected snails contained rediae with developing cercariae. Sporocysts were not observed. Comparison with the description of *A. progenetica* as given by Serkova and Bykhovskii (1940) and other described species disclosed differences which led the authors to designate their specimens as members of a new species, *Asymphylodora dollfusi*. Observations on the developmental cycles of *Asymphylodora tincae* and other digenetic trematodes were reported by Wisniewski (1956).

Observations on abbreviated life-cycles have been recorded also for members of other taxonomic groups. Progenetic metacercariae of hemiurid species were reported from copepods by Dollfus (1954) and by Chabaud and Biguet (1954). Adult specimens of another hemiurid were reported from *Sagitta elegans* by Betty Myers (1956). Crusz (1956) reported progenetic metacercariae of *Cercaria patialensis*, a species described by Soparkar (1924) from *Melanoides tuberculata* and related to *Transversotrema* by Witenberg (1944). Peters (1957) described *Allocreadium neotenicum* n. sp., from sexually mature specimens found in water beetles. Stunkard and Uzmann (1959) reported that sexually mature and gravid worms found in *Mytilus edulis* are specifically identical with *Cercaria milfordensis* Uzmann, 1953 and with *Proctoeces maculatus* (Looss, 1901). Freeman and Llewellyn (1958) described adult worms in the renal organs of the lamellibranch mollusk, *Scrobicularia plana*, taken from the flats of the Thames estuary, at Chalkwell in Essex and Whitstable in Kent. The specimens were identified as *Proctoeces subtenuis* (Linton, 1907), which Stunkard and Uzmann (1959) synonymized with *P. maculatus*.

The extent and incidence of progenetic maturity in larvae of digenetic trematodes have aroused questions in the minds of many investigators since Dollfus posed the problem. After reporting sexual maturity in metacercariae of a species of *Clinostomum*, McIntosh (1935, p. 79) wrote, "Do these records of precocious development throw light on what the adult flukes were like in the ages before the vertebrates came into being, or may the phenomenon be explained on the basis of the similarity of the environment of the metacercaria with that of the habitat of the adult?" Buttner (1951) attempted to evaluate the environmental factors which might affect sexual development and raised the question whether or not

progenesis might provide data concerning the origin of parasitism among the trematodes; whether it represents successive stages in the evolution of existing life-cycles or is merely an abridgment or simplification of otherwise more complicated cycles. She was inclined to regard progenesis as the result of a mutation in hereditary factors rather than as the result of environmental influences. Szidat (1956) discussed the causes and meaning of abbreviated life cycles in trematodes and cestodes. He stated that formation of eggs in the first intermediate host would undoubtedly be favorable for preservation of the species and that the addition of intermediate hosts might be explained as steps in the elaboration of a food-chain.

The origin of metagenesis, alternation of sexual and asexual generations, has received the attention of biologists for more than one hundred years. It was first recognized by Steenstrup in coelenterates and parasitic flatworms. Actually, the phenomenon was initiated and perfected in plants. In the early amphibious plants, liverworts and mosses, the sexual gametophyte was dominant and the asexual sporophyte arose as a microscopic and parasitic generation when germinal cells separated from the parent organism and, enclosed in a protective covering, proved capable of developing new gametophytes. It is probable that the development of the sporophytic generation was an adaptation to terrestrial existence. In the course of evolution, as represented by the pteridophytes, both generations were independent with the sporophyte gradually assuming the ascendancy, while in seed-bearing plants the asexual sporophyte became dominant with the sexual generation reduced to a few cells, entirely dependent and parasitic on the asexual one. In plants, the gametophyte is haploid and the sporophyte is diploid, with reduction of chromosomes at sporogenesis. In the Coccidia and other sporozoans, reduction is zygotic, whereas in the trematodes both generations are diploid and reduction is gametic. It appears, therefore, that the location in the life-cycle where the reduction divisions occur is not determinate in the evolution of metagenesis.

Among endoparasites, the parasite perishes with the host, and many species which became successful developed accessory, *i.e.*, asexual types of reproduction and provision for emergence and dispersal of the asexually produced individuals. The introduction of asexual methods was essentially similar in plants and animals. In both they evolved in a parasitic generation. In the protozoans which became cellular or histozoic parasites, sporulation became the characteristic method of reproduction with alternation of generations. Sporulation involves rapid nuclear divisions in a common cytoplasmic matrix, the formation of a plasmodium, and subsequent multiple fission with the production of many new individuals. In the asexual cycle, each cell which results from sporulation is termed a merozoite. Certain of these cells, usually after repeated divisions and the development of physiological maturity, become gametocytes and produce gametes. In the sexual cycle, the fusion of gametes produces a zygote which again undergoes sporulation and the production of sporozoites. Both types of reproduction may take place in the same host or, in other species, alternation of generations and hosts may occur.

The parasitic flatworms were derived from free-living species by gradual and progressive adaptation and present life-cycles are not primitive. The Digenea are distinct in morphology, bionomics and life-cycles from the Monogenea and

the origin of double hosts with alternation of generations, involving sexual and asexual types of reproduction, is still an unresolved problem. It is generally agreed that the Digenea have a common ancestry with the Turbellaria. In certain rhabdocoels, asexual reproduction regularly alternates with sexual reproduction. It appears that asexual reproduction is associated with rapid metabolism and a juvenile condition and that sexual maturity is attained with physiological maturity. The turbellarians are primitively carnivorous and certain dalyellioid species, probably related to the ancestors of the Digenea, are parasitic in mollusks and echinoderms. Others are commensals or pseudoparasites. Leuckart maintained repeatedly that the mollusk was the original host of the early digenetic trematodes and his argument was indorsed by Stunkard (1957).

It is accepted that endoparasitism frequently results in the acquisition of accessory, asexual methods of reproduction. If the ciliated larva of the turbellarian, on invasion of a mollusk, were to form a cyst-like structure in which the germinal cells were to separate and develop independently, a very efficient method of reproduction, polyembryony, would have arisen. The repeated dissociation of germinal cells could continue to form daughter sporocysts, but the progeny would tend eventually to develop turbellarian structures, notably a pharynx and simple gut, and a redia-like individual would result. Such a sexually mature stage may be discerned in members of the Aspidogastridae.

Polyembryony in sporocysts and rediae would produce population pressure and individuals would be expelled from the host. The finding of a new host required locomotion and the modification of the posterior portion of the body to form a device for sculling through the water gave a distinct advantage over mere wriggling movements or the successive attachments of suckers. The diversity in types of cercarial tails indicates the many directions in which this adaptation has progressed. The asexually produced individuals, on leaving their former hosts, would be dispersed throughout the neighboring area. Such worms, already pre-adapted for parasitic existence, would invade and become sexually mature in other animals, and in essential respects could be compared with present-day progenetic metacercariae. With the advent of vertebrates, these parasites together with their hosts were eaten; as a result the worms were introduced into new hosts. With establishment in the newly acquired vertebrate hosts, sexual maturity was naturally more and more deferred to worms in the definitive hosts. Meanwhile, the former definitive hosts became secondary intermediate, paratenic or transport hosts, providing for completion of the newly acquired life-cycle. The vertebrates, wider-ranging and longer-lived animals, would facilitate the dispersal and prolong the life of the parasites. After reviewing life cycles of parasitic worms in the different classes, Stunkard (1953) concluded, ". . . it is apparent that developmental cycles have been evolved concomitantly with the parasitic habit, that they are bionomic adaptations to ecological conditions, and that modifications in the course of the life-cycle were introduced accidentally and have been perpetuated because they facilitated dispersal and had survival value for the species." Progenesis occurs in members of many families; its distribution and incidence are too extensive to be explained as a mutation; rather it appears to be a relict, the persistence of an earlier state, which obtained before sexual maturity was so largely deferred to definitive vertebrate hosts.

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STUDIES ON THE PHYSIOLOGICAL VARIATION BETWEEN TROPICAL AND TEMPERATE ZONE FIDDLER CRABS OF THE GENUS UCA. III. THE INFLUENCE OF TEMPERATURE ACCLIMATION ON OXYGEN CONSUMPTION OF WHOLE ORGANISMS[1]

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As organisms extend their distributional limits, they are faced with new environmental stresses. Through the mechanism of natural selection, populations of animals have arisen which show varying degrees of physiological adaptation. Review papers by Prosser (1955) and Bullock (1955) have excellent discussions of many aspects of this subject.

The present series of papers deals with physiological variation between temperate and tropical zone species of fiddler crabs (genus *Uca*). Results of the first paper (Vernberg and Tashian, 1959) demonstrated the marked difference in thermal death limits, especially at low temperatures, of latitudinally isolated populations of fiddler crabs. The 50% mortality level reached after 30–40 minutes at 7° C. for *Uca rapax* was characteristic of the other fiddler crabs studied in Jamaica, The West Indies, while temperate zone species from North Carolina lived for weeks at this temperature. Acclimation to reduced temperature (15° C.) had little effect on the response of tropical species but greatly increased the survival time of temperate zone forms. The second paper (Vernberg, 1959) dealt with the oxygen consumption of fiddler crabs as influenced by temperature, season, size and starvation. The seasonal variation in rates of oxygen consumption observed in *U. pugnax* from North Carolina, but not in the similar sized crab, *U. rapax* from Jamaica, suggested that temperate zone crabs possessed a labile respiratory mechanism while the response of tropical species is fixed within narrow limits.

The importance of temperature acclimation studies in understanding the metabolism of latitudinally isolated populations has been stressed for some time. Therefore the present investigation was undertaken to study in more detail the role of acclimation in determining metabolic differences between tropical and temperate

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zone species. Since this study began, papers on crab respiration by Démeusy (1957), Roberts (1957) and Teal (1959) have further emphasized the need for additional work on this problem.

Materials and Methods

In the present paper two species of *Uca* were selected, *Uca pugnax* (Smith) from North Carolina and *Uca rapax* (Smith) from Jamaica and Florida. These particular species were chosen for a number of reasons: both species are closely related; until recently (Tashian and Vernberg, 1958), both species were considered races belonging to one species, the southern form being *U. pugnax rapax* and the northern race *U. pugnax pugnax*. Populations of these two species are found on the coast from Massachusetts to Brazil with a zone of overlap along the northeast coast of Florida. Thus they represent an excellent continuous series for a comparative study. Both species not only are available in great numbers, but they are easily maintained under laboratory conditions. Results of acclimation studies could be correlated with detailed metabolic data already available on these two species (Vernberg, 1959).

Experimental studies on fiddler crabs from North Carolina and Florida were conducted either at the Duke University Marine Laboratory or at Duke University, and the tropical species was studied at the University College of the West Indies, Jamaica.

The method of determining oxygen consumption, as well as the procedure for the laboratory care of animals, was described in a preceding paper (Vernberg, 1959). All results are expressed as cu. mm. oxygen consumed/minute/gram of animal (wet weight). Preliminary studies showed that the time interval necessary for thermal equilibration and for the rate of oxygen uptake to reach a somewhat steady level varied inversely with temperature. Only oxygen consumption data which were relatively stable over a period of time were used. An attempt was made to minimize variation due to rhythmic daily fluctuations by making an equal number of determinations in the afternoon and the morning. No attempt was made to correct for possible tidal influence on metabolism. Although the determinations were made at different times of the year, the recent thermal history of animals from North Carolina and Jamaica was the same in that their habitat and laboratory temperatures were alike. Work at Beaufort extended from June to September, while studies in Jamaica were from October, 1957, to April, 1958. Animals from Florida were collected in October and November and maintained in the laboratory until needed.

Animals subjected to 15° C. for various periods of time are referred to as cold-acclimated or "cold" animals, while animals maintained at room temperature (22–27°) will be called warm-acclimated or "room" animals.

Results

Uca pugnax from Beaufort, North Carolina

The metabolic response of 21 warm-acclimated *U. pugnax* was determined at 27° C. and then these animals were kept at 15° for 21 days. Throughout the period

of exposure to reduced temperature the rate of oxygen consumption was re-determined at 27° C. at specific intervals of time. Another group of animals, which were maintained in a starved condition at room temperature, were used as a control. Thus the per cent change in metabolic rate shown in Figure 1 represents shifts in rate of oxygen consumption relative to control animals as 0% change.

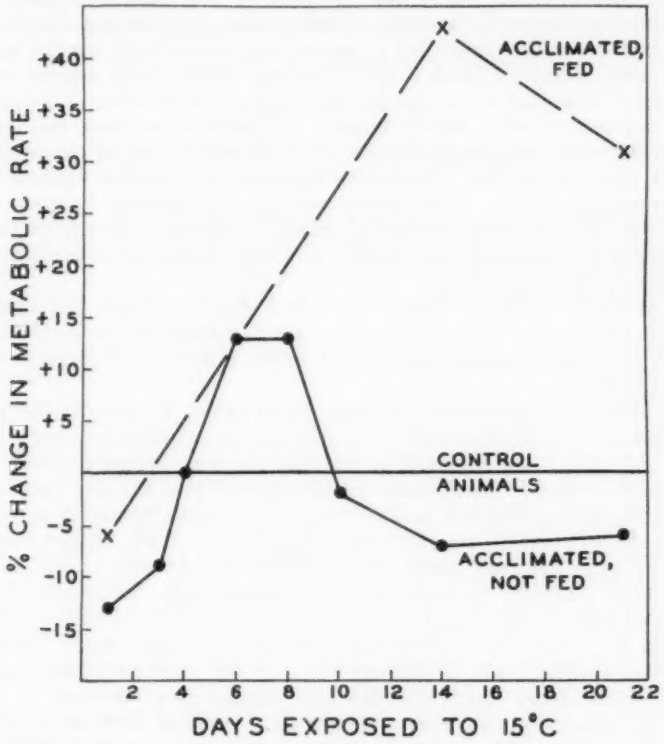

FIGURE 1. The influence of starvation during acclimation to 15° C. on the respiratory metabolism of *Uca pugnax* determined at 27° C. Percentage relationship of changes in rate of oxygen consumption relative to control animals as 0% change.

Their metabolic rate, which was initially depressed by cold, increased by day 6. The rate at day 10 was lower and fluctuated slightly during the remaining 11 days. A tendency to acclimate was apparent until days 6–8 and then was lost.

To determine the role of food in acclimation, this same experiment was repeated with one variation: food was presented to the 15 animals maintained at 15° C. on day 7 and day 11, while the rate of oxygen uptake was determined on days 1, 14, and 21. In a previous paper (Vernberg, 1959), it was shown that their metabolic rate dropped markedly after one day of starvation. This trend

continued until day 3–5 when a leveling-off occurred with minor fluctuations observed until the end of the experiment on day 21. Thus in the present study, it was felt that the metabolic rate determined on day 14 did not reflect post-absorptive effects. Results, which are included in Figure 1, show that food had a pronounced influence on metabolism during temperature acclimation. The metabolic rate did not decline after day 6–8 as observed in the unfed animals, but continued high until the termination of the experiment on day 21.

Table I represents oxygen consumption data on animals which had been acclimated to 15° for various periods of time. The per cent change in metabolism is relative to values obtained for control animals. In this series of determinations, control animals are defined as warm-acclimated animals whose metabolic

TABLE I

The influence of acclimation to 15° C. for various periods of time on the respiratory metabolism of Uca pugnax determined at different temperatures. Percentage relationship of changes in rate of oxygen consumption relative to control animals as 0% change

Temperature °C.	No. of days at 15°	% change
7	1	+21
7	21	+37*
17	1–2	+24**
17	11	+11
17	21	+16
27	1	−13
27	6	+13
27	14	+43*
33	1	− 8
33	9–11	+ 4
33	18–21	− 3
39	1	− 9
39	11	− 1

* Mean is significantly different from control at 1% level.
** Mean is significantly different from control at 5% level.

rate had been determined at various thermal levels. In all cases, these animals are starved for three to five days before determinations are made. For example, animals acclimated to 15° for 21 days consume oxygen 37% faster than warm-acclimated fiddler crabs when determined at 7° C. A significant difference in metabolic rate during cold acclimation was observed only at a few points. At 7° a marked increase was noted within 24 hours, but it was not until 21 days that the difference in the rate of oxygen consumption was significant at the 1% level. Although individuals exposed for 11 days or 21 days used oxygen at a faster rate than room-animals when determined at 17°, significant differences (at the 5% level) were observed only with 1 day animals. Results obtained at 27° were discussed above. Slight differences were observed at 33° and 39°.

When determined at low temperatures (7° and 17°) the metabolic rate of

cold-acclimated animals was always higher than control animals, but at elevated temperatures (33° and 39°) the reverse was observed (Fig. 2). At an intermediate temperature of 27° the response was intermediate; first the rate was depressed, as was the response at elevated temperatures, and then the rate increased with exposure to cold, much like the response at lower temperatures. The points on Figure 2 for cold-acclimated animals at 7°, 17° and 27° represented the maximum respiratory response to cold.

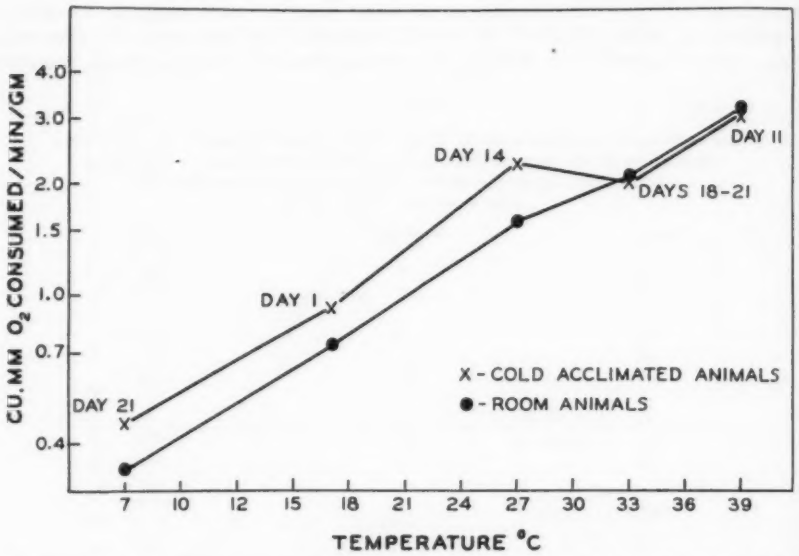

FIGURE 2. The oxygen consumption at various temperatures of room-animals and cold-acclimated *Uca pugnax* from Beaufort, North Carolina. Cold-acclimated animals had been kept at 15° C. for the periods of time indicated.

Uca rapax from Jamaica

The metabolic rate was determined at 28°, the animals were subjected to 15° for specific intervals of time, and then their metabolic rate determined again at 28°. At the beginning of this experiment 26 animals were used, but this number decreased with time due to death or discarding of animals which had lost appendages: three animals lost their large chela, two animals were dead after three days at 15°, five more had died by the fifth day, and three additional animals had died by day 7. No determinations were made after day 7 as the mortality level was too high. As a more critical method of analysis, the averages for the different days were computed only on the animals surviving throughout a particular time interval, *i.e.*, by day 5 only 18 animals survived, thus the averages cited in Table II reflect the data on only these 18 animals. Results of this experiment show that significant variation was observed only after one-day exposure to 15° and then

only at the 3% level. It should be noted that in Table II the figures for the average metabolic index are not in terms of cu. mm. of O_2/min./gram as were the data on *U. pugnax*. These figures represent the average of the total number of manometer units change divided by the total elapsed time in minutes. For each successive daily determination an animal was placed in the same flask. Thus relative values could be used, and it was not necessary to multiply by the flask constant and divide by the weight of the animal to determine the absolute metabolic rate.

TABLE II

The influence of acclimation to 15° for various periods of time on the respiratory metabolism of Uca rapax from Jamaica when determined at 28°

Number of animals	Number of days at 15°	Average metabolic index	S.E.*
26	0	0.504	0.063
26	1	0.676**	0.048**
23	0	0.518	0.070
23	3	0.544	0.043
18	0	0.504	0.070
18	5	0.577	0.038
13	0	0.484	0.065
13	7	0.523	0.070

* S.E.—Standard Error.
** Means are significantly different at 3% level.

When determining the rate of oxygen consumption at 15° and then after exposure to 15° for one and five days, no significant fluctuation was observed. Similar rates of oxygen uptake determined at 12° were noted for 25 animals kept at room temperature and for 17 animals exposed to 15° for five days. Although temperature acclimation produced no apparent modification in metabolism when determined at low temperatures, marked changes were observed at higher temperatures. After five days at 15°, 19 cold-acclimated crabs consumed oxygen at a significantly higher rate than 26 animals maintained at room temperatures (at the 1% level) when determined at 36° C. These results are graphically represented in Figure 3.

Uca rapax from Alligator Harbor, Florida

Table III presents data on the metabolic response of *Uca rapax* from Alligator Harbor, Florida, after exposure to 15° for various periods of time. Unlike Jamaican forms, but similar to the more northern species, a significant drop (at the 1% level) in metabolic rate resulted after 24 hours at this reduced temperature. By days 3 and 8 no significant difference between non-acclimated and acclimated animals was noted. However, further exposure to cold resulted in a decreased rate which by days 12–14 was significant at the 5% level. Mortality was prac-

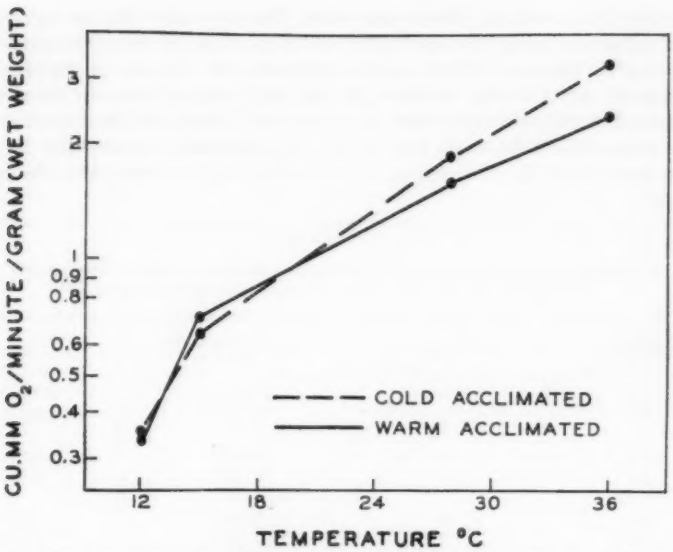

FIGURE 3. The oxygen consumption at various temperatures of warm- and cold-acclimated *Uca rapax* from Jamaica, The West Indies.

TABLE III

Metabolic response of Uca rapax from Alligator Harbor, Florida, after exposure to 15° C. for specific periods of time. Determinations made at various temperatures and results expressed as cu. mm. of O_2 consumed/minute/gram of wet weight

Temperature °C.	No. days at 15°	Rate of oxygen consumption and standard error	No. of determinations	Significance of difference of means
35	0	2.901 ± 0.149	31	1% level
	11–14	2.026 ± 0.177	16	very significant
28	0	1.916 ± 0.087	58	3% level
28	1	1.695 ± 0.051	58	significant
28	0	1.856 —	36	not significant
28	3	1.666 —	36	
28	0	2.017 ± 0.102	26	not significant
28	8	1.802 ± 0.085	26	
28	0	2.438 ± 0.102	36	5% level
28	12–14	2.144 ± 0.108	36	significant
17	0	1.017 ± 0.066	30	5% level
17	29–53	1.217 ± 0.073	30	significant
7	0	0.352 ± 0.019	24	1% level
7	33–54	0.460 ± 0.011	16	very significant

tically negligible during this two-week period, which is in sharp distinction with the response of tropical forms.

Figure 4 represents the mean metabolic rate of cold-acclimated and room-animals determined at various temperatures. The means are significantly different for these two groups of animals at the four temperature points studied. At 7° and 35°, significance is at the 1% level, while at 17° and 27°–28° significance is at the 5% level. Determinations at 7° and 17° were made on animals maintained at 15° for 29–53 days. No apparent difference was observed in the response of 29 day or 53 day animals; this suggests that acclimation is complete

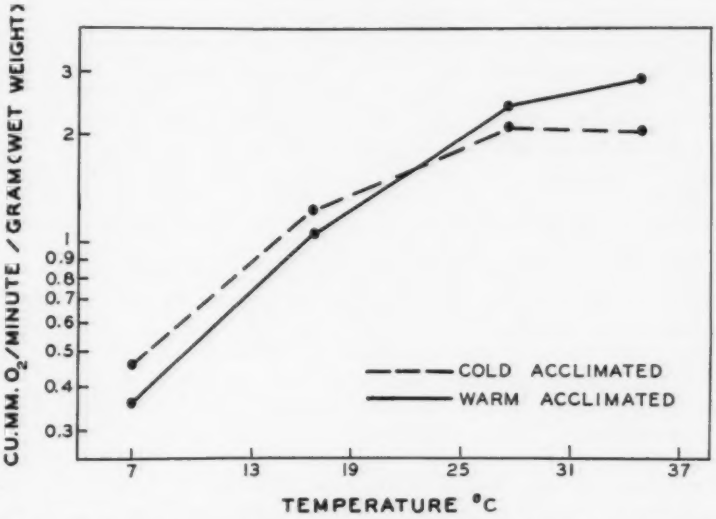

FIGURE 4. The oxygen consumption at various temperatures of warm- and cold-acclimated *Uca rapax* from Alligator Harbor, Florida.

within this time. At 27°–28° and 35°, animals had been kept for 11–14 days. Although greater metabolic differences might have been observed if animals had been maintained at 15° longer, it was felt that on the basis of work on *Uca pugnax* from North Carolina this period of time was sufficiently long to produce marked changes in metabolism.

DISCUSSION

Results of this study on the influence of thermal acclimation on respiratory metabolism furnish further evidence of pronounced physiological differences between temperate and tropical zone fiddler crabs. This work is in general agreement with the findings of other workers in this field.

Roberts (1957), working with population samples of *Pachygrapsus crassipes* collected from southern California to Oregon, found a close correlation between compensatory respiration levels and habitat temperature during the winter. North-

ern crabs consumed oxygen at a faster rate than southern forms when determined at an acclimation temperature higher than the field temperature at the time of collection. Although the experimental conditions in the present study are different from those of Roberts (1957), the same general tendency exists for individuals of the northern population to exhibit higher metabolic rates at low and intermediate temperatures than southern members of the same species. Roberts worked with a species inhabiting the temperate zone and thus subjected to marked seasonal temperature fluctuations, while the *Uca rapax*, used in the present study, extends from the tropics, where the temperature varies slightly during the year, to northern Florida with its seasonal thermal changes. Therefore, it is not surprising to note that the pattern of response of *U. rapax* from Florida is not like the tropical crabs, but more similar to that of *U. pugnax* from North Carolina, especially at temperatures below 17°. It would appear that with a selection pressure for temperature adaptability, a similar metabolic pattern has evolved in temperate zone forms. Although the response of these organisms might be similar, as measured by the oxygen consumption of the whole animal, the mechanisms governing the utilization of oxygen by the cells in these two species may be entirely different.

Uca pugnax appeared to require two weeks to be completely acclimated to low temperature (15°), which shows remarkable similarity to results of Roberts. When comparing these two papers it should be kept in mind that the absolute temperature referred to is not of paramount importance; the relative response of the organisms is the major consideration. In the study of *Pachygrapsus* the temperature (16°) used to base the degree of acclimation to warm or cold was close to the environmental mean for this species. However, in North Carolina the mean habitat temperature of *U. pugnax* during the summer is about 27°.

In both species, *U. pugnax* and *P. crassipes*, cold-acclimated forms consumed oxygen at a faster rate than warm-acclimated animals when determined over a wide temperature range. The only difference was that at elevated temperatures cold-acclimated *U. pugnax* consumed oxygen at the same rate as room-animals, whereas cold-acclimated *P. crassipes* continued to show greater respiratory activity than warm-acclimated forms. Although this difference in response is difficult to explain, it may be associated with the differences in environmental temperature extremes encountered by the two species. At Beaufort, North Carolina, the mean monthly water temperature varies from 5.5° in February to 28° in August (McDougall, 1943), while data presented by Roberts show yearly changes of a low of about 13° to a high of about 18° for Point Hueneme, California.

Working with *U. pugilator* from Massachusetts and Florida, Démeusy (1957) measured their respiratory rates at 1.4° and 15° after being maintained at 20° for various time intervals. The northern forms showed higher rates throughout a seven-week period when determined at 1.4°. Although no statistically significant difference was noted between the respiratory activity of the two groups of fiddler crabs at 15°, it should be noted that determinations were not made at this temperature over an extended period of time. In the present study, northern forms of *U. rapax* consumed oxygen at a higher rate than southern forms, especially at lower temperatures, which is similar to the findings of both Démeusy and Roberts.

In contrast to the results of Roberts, Démeusy found no decrease in metabolic rate of crabs from different localities when kept at a common temperature. She felt that regular feeding of the animals might be responsible for this steady rate of metabolism. Results of the present paper demonstrate the following: (1) feeding of *U. pugnax* during acclimation to 15° resulted in the maintenance of a steady rate of oxygen consumption after two weeks; and (2) the temperate zone forms of *U. rapax* showed a gradual decline in metabolic rate during acclimation to 15°, as shown by Roberts, while the tropical animals showed a tendency to have higher rates.

The respiratory activity at 1.4° of *U. pugilator* from Florida decreased slightly for the first seven days of acclimation to 10° when compared with crabs kept at room temperature. This decrease was followed by a slight increase after one week. Although *U. rapax* from Florida and *U. pugnax* were subjected to slightly different experimental conditions, cold-acclimated animals of both species consumed oxygen at significantly higher levels than room-animals when determined at 7°. This response is similar to findings of Roberts on *Pachygrapsus*. However, the respiratory pattern of the tropical form of *U. rapax* did not change during acclimation to 15° except at elevated temperatures.

Recently Teal (1959) reported that on the basis of studies on the crabs found in one area, a Georgia salt marsh, *U. pugnax* had a better developed mechanism for thermal acclimation than *U. pugilator*. This might help explain some apparent differences between the results of Démeusy and those of the present paper.

Prosser (1958) has described various patterns of temperature acclimation based on measuring reaction rates over a range extending beyond the acclimation temperature. Using his terminology, it is possible to characterize the pattern of response of the tropical and temperate zone fiddler crabs used in the present study.

Tropical forms of *U. rapax* appear to best fit pattern I (no adaptation) except between 28° and 36° where the Q_{10} of cold-acclimated animals is increased significantly over that of warm-acclimated organisms, suggesting pattern IIIB. Similar results have been reported by Scholander *et al.* (1953), when comparing some tropical and arctic terrestrial insects. The metabolic response of the temperate zone form of *U. rapax* appears to resemble pattern IIIA, which indicates that the Q_{10} of cold-acclimated animals is less than the Q_{10} of warm-acclimated forms. Démeusy (1957) found this response in *U. pugilator*. *U. pugnax* from North Carolina seems to fit pattern IVA best, which seems to be the most common method of temperature acclimation, according to Prosser. Rao (1953) observed this pattern in the rate of pumping water by *Mytilus* from different latitudes.

On the basis of this method of comparing patterns of metabolic response during temperature acclimation, certain generalizations might be made. Both populations of *U. rapax* demonstrated a rotation of rate function curves, whereas in *U. pugnax* the type of change is a translation of curves. This might suggest that different biochemical mechanisms are involved in these two species.

The results of Tashian (1956) on oxygen consumption of *Uca* from different latitudes indicate that different patterns may be found when making intra- and inter-specific comparisons of animals from different geographic locations. This was noted also in the present study. Intraspecific comparisons of *U. pugnax*

from New York and North Carolina and *U. rapax* from Florida and Trinidad represent pattern IIA. An inter-specific comparison of *U. pugnax* from New York and *U. rapax* from Trinidad and Florida suggests pattern IIIA, while comparing *U. pugnax* from North Carolina and *U. rapax* from Florida seems to indicate pattern IVB. It should be noted that this work was done on animals with similar recent thermal histories and determinations were made at two temperatures. Vernberg (1959) measured the rate of oxygen consumption over a wide temperature range of 7 species of temperate and tropical zone fiddler crabs. He noted that systematic differences correlated with latitude were found only at lower temperatures. The findings of the present paper, which involved acclimation studies, suggest that with the evolution and distribution of animals to new environmental complexes a new pattern of acclimation has resulted.

Summary

1. Studies on the metabolic response of tropical and temperate zone fiddler crabs (Genus *Uca*), after various periods of thermal acclimation, were done to assess the importance of this factor in the evolution and climatic adaptation of these forms.

2. *U. pugnax*, a temperate zone species, showed a significantly higher metabolic rate after acclimation to 15° than warm-acclimated forms when determined at 27°, 17° and 7°, but not at 33° and 39°. When animals were starved during the period of acclimation, the tendency to acclimate was apparent by days 6–8 and then was lost. Feeding maintained the pattern of acclimation for at least 21 days.

3. *U. rapax* from Jamaica, The West Indies, did not show any shift in metabolic response during acclimation to 15°, except when determined at 36°, where the rate of cold-acclimated forms was higher than warm-acclimated animals.

4. Population samples of *U. rapax* from Florida responded metabolically like temperate zone animals at low temperatures and the tropical animals at high temperatures.

5. Results of this study demonstrated that during the evolution and distribution of fiddler crabs different patterns of acclimation have resulted.

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RESPIRATION AND ANAEROBIC SURVIVAL IN SOME SEA WEED-INHABITING INVERTEBRATES [1, 2]

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L. C. Beadle in a paper on the respiration in the African swampworm *Alma emini* (Beadle, 1957) showed that the waterlogged mats of *Cyperus papyrus* are devoid of oxygen most of the time and that all animals living in them have to be adapted to this situation one way or the other. The case of the swamps is certainly an extreme one, but there can be no doubt that wherever plants grow in water or are easily flooded, anaerobic conditions may prevail occasionally or quite regularly.

The large sea weeds growing along the open coasts seem to represent an exception to such a statement and might be regarded as the prototype of a well-aerated habitat. This is certainly true at high tide. At low tide, however, these sea weeds collapse, forming thick clumps within and under which water is trapped. In the night or on cloudy days respiration of the algae may exceed photosynthesis and the water, cut off from atmospheric circulation, will gradually lose its oxygen to the plants.

This, then poses an ecological problem which can be stated as follows: Does the oxygen content of the water trapped by emersed sea weeds ever fall to zero and if so, how do the animals living in the sea weeds cope with this situation?

Material and Methods

The work was carried out at the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts. The water samples were taken from under clumps of emersed brown algae (*Fucus, Ascophyllum*) on July 13 and 14, 1959, by means of 5-cc. syringes which were stoppered with glass plugs, and analyzed in the laboratory with the gasometric water analyzer of Scholander (Scholander *et al.*, 1955). The sampling site was a flat piece of gravelly and rocky beach (Woods Hole Yacht Club) on which brown algae grew quite luxuriantly. All the samples came from the upper and the middle zone of the exposed algal belt. The tidal range at Woods Hole is 1.8–2.2 ft. The temperature within a clump of sea weeds measured 20° C. at one occasion.

Various animals living in the large brown sea weeds around the Woods Hole Oceanographic Institution were collected at high tide and their respiration and anaerobic survival studied. The respiration of nematodes and mites was measured by the Cartesian diver method as described by Holter and Linderstrøm-Lang (1943) and others. The divers used had a volume from 6.5 to 37.4 μl. and volume changes could be read to 0.001–0.006 μl. O_2/hr. The respiration of the

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[2] Contribution no. 1061 from the Woods Hole Oceanographic Institution.

amphipods was measured by means of the volumetric micro- and macro-respirometer of Scholander (Scholander *et al.*, 1952). Respirometer runs were made at 10° C. and in the following diagram the respiration values are corrected to 20° C., the temperature at which the diver was run, by assuming a Q_{10} of 2. This value seems to be a reasonable—perhaps even too low—guess in the light of the data on the amphipod *Talorchestia* by Edwards and Irving (1943) as re-plotted by Rao and Bullock (1954).

Anaerobic survival was studied by putting the animals into a small amount of water in a Thunberg tube which then was evacuated and flushed with tank-nitrogen. Constant experimental conditions were maintained by putting a constant amount

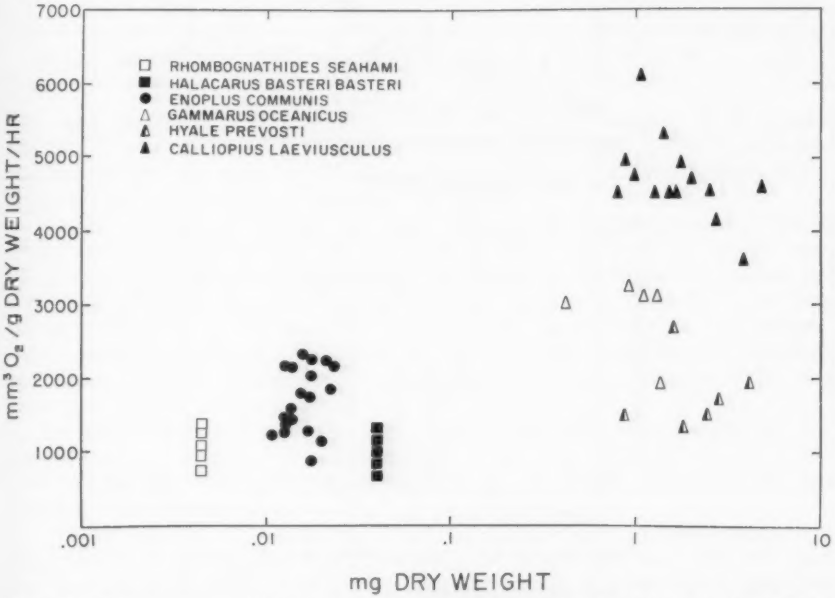

FIGURE 1. Oxygen consumption per gram dry weight of the six species of animals investigated. Each convention represents one experiment.

of water into the Thunberg tubes and by flushing each tube exactly 100 times. The evacuation of the Thunberg tubes was carried out at room temperature. Subsequently, the tubes were kept either in the refrigerator at approximately 2° C., or at 15° C., or in the laboratory which had a fairly constant temperature of 25° C. The behavior of the animals was studied either by observing them in the closed tube, or after a certain time had elapsed, by emptying them into a dish with normal sea water. The recovery time of nematodes and mites was defined as the time interval within which 75% of the animals used for one experiment had resumed movement.

The dry weight of the nematodes was established by measuring volume and specific gravity as described elsewhere (Wieser, 1960). The dry/wet weight ratio

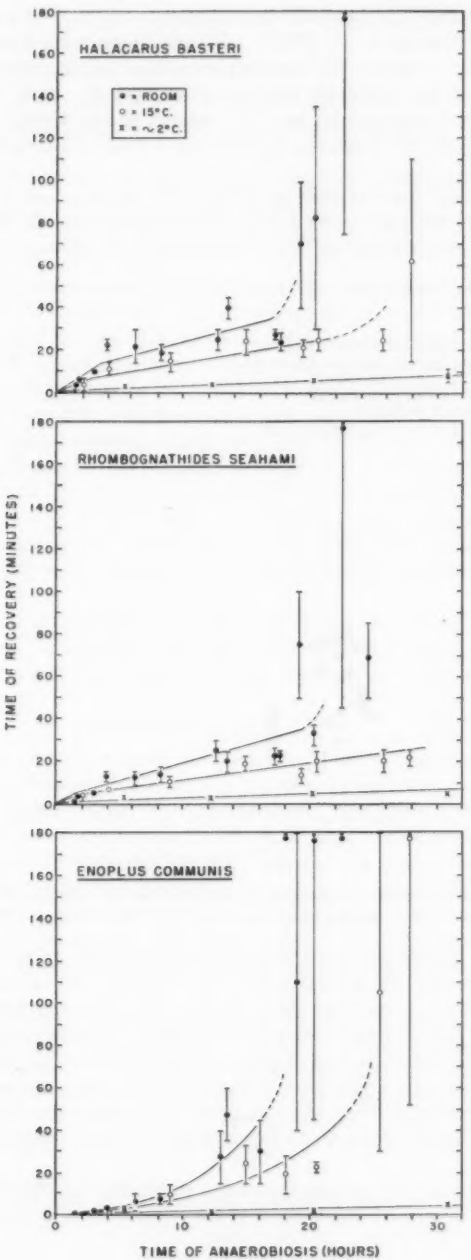
HALACARUS BASTERI
● = ROOM
○ = 15°C.
x = ~2°C.
RHOMBOGNATHIDES SEAHAMI
ENOPLUS COMMUNIS
TIME OF RECOVERY (MINUTES)
TIME OF ANAEROBIOSIS (HOURS)

FIGURE 2.

was assumed to be 25% in all specimens. The mites were dried at 100° C. and weighed in batches on a "Misco" quartz helix (by Dr. R. Conover, W.H.O.I.). The dried amphipods were weighed individually on a torsion balance.

Results

The water analyses gave the following results:

Sample no.:	1	2	3	4	5	6
Time:	10 P.M.		10 A.M.			
Ml. O_2/1 liter H_2O:	0.25	0.30	0.0	0.05	0.96	1.65

The night samples were taken exactly at low tide, the day samples 30–40 minutes after low tide on a cloudy day (illumination 1700 foot-candles). The oxygen content of saturated sea water at 20° C. and the local salinity (approximately 31‰) is 5.5 ml. O_2/1 liter H_2O (Sverdrup *et al.*, 1946).

For the measurement of respiration and anaerobic survival two groups of species were chosen; one, consisting of the nematode *Enoplus communis* and the sea mites (Halacaridae) *Rhombognathides seahami* and *Halacarus basteri basteri*, represented very small animals of low motility, unable to swim and without special respiratory organs. The other group consisted of three amphipod species, *Calliopius laeviusculus, Gammarus oceanicus* (identified by Dr. H. Werntz, Harvard University) and *Hyale prevosti*, representing medium-sized, very agile animals with good swimming capabilities and equipped with gills.

The respiratory rates per gram dry weight of the six species investigated are summarized in Figure 1. It is evident that if the size difference is taken into account (Zeuthen, 1947), the amphipods have a much higher metabolic rate than the other three species.

The two groups of species could also be distinguished in their reaction to anaerobic stress. Nematodes and mites, after the oxygen had been removed from the Thunberg tube, became paralyzed. From this state they recovered after they were put back into oxygenated water, provided that the time of anaerobic stress had not lasted too long. The recovery time as shown in Figure 2 is a function of the time of anaerobiosis and of the temperature at which the latter was spent. The three amphipods, however, remained active under anaerobic stress for a certain time and then died (Fig. 3). One could, of course, say that they, too, became paralyzed after a period of activity, but that the paralyzed state which preceded death was of extremely short duration. If an animal after readmission to normal conditions did not recover right away, it did not recover at all.

Figure 2. Relationship between time of anaerobiosis and time of recovery in the two halacarids (upper two diagrams) and the nematode. Each convention represents one experiment on a batch of animals. Range of variability in recovery time indicates beginning of movements in the first and in 75% of the animals in one batch. Recovery periods exceeding 180 minutes are characterized by an arrow and by a circle close to the upper abscissa of the diagram.

The survival period of one amphipod, *Calliopius laeviusculus,* was extremely short, certainly not more than 5 minutes at a temperature of 25° C. The other two species, *Gammarus oceanicus* and *Hyale prevosti,* did considerably better, the survival period ranging from about half an hour of anaerobic stress to about three hours. There was no clear-cut relationship between survival time and the size of the animals.

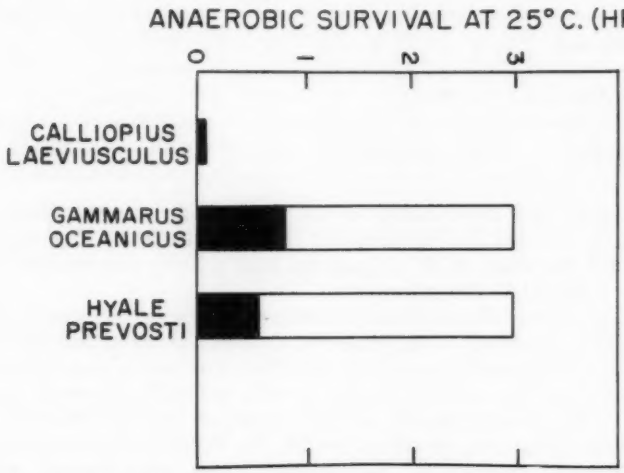

FIGURE 3. Survival under anaerobic conditions of the three species of amphipods studied. The black part of each column indicates the time of anaerobiosis *all* individuals survived, the white part indicates ranges of variability of survival time.

DISCUSSION

The water analyses show plainly that animals living in the water trapped by large sea weeds at low tide may be subject to oxygen-free or almost oxygen-free conditions. Under extreme circumstances (very large clumps of algae, no light, high temperature) these conditions probably set in soon after the receding tide has uncovered the plants and may last for several hours. A drop in the oxygen content to 30% saturation value was found by Revelle and Emery (1958) in intertidal basins with algal growth at the bottom.

Animals living in sea weeds react in different ways to the movements of the tides. As shown previously (Wieser, 1952), some animals remain in the sea weeds all the time while others (in Plymouth, England, *e.g.* copepods and the amphipod *Stenothoe monoculoides*) leave their habitat with the receding tide and return to it with the rising tide. To the former group, obviously, belong slow-moving, non-swimming animals like the nematode and the mites studied in this paper. These species are adjusted to their environment by being able to withstand anaerobic conditions at 25° C. for at least 12 hours which, in an area with diurnal tides, is the theoretical maximum their habitat could remain emersed.

That paralysis during anaerobiosis is due to the accumulation of toxic metabolites within the body has been pointed out by von Brand (1946). Since the accumulation of metabolic substances is a temperature-dependent process, the close dependence of the rate of recovery from paralysis on the temperature at which the anaerobiotic period was spent (Fig. 2) could have been anticipated (see also Miller, 1957). Furthermore, the recovery time from anaerobiosis is a characteristic of each species. In the two halacarids, a linear relationship exists between the time of anaerobiosis and the time of recovery, up to the point at which the effects of asphyxiation become lethal and the curve expressing the relationship turns sharply upwards (Fig. 2). The slope of the linear portion of the curve is steep in the beginning and flattens out somewhat after 2–4 hours' application of anaerobic stress at room temperature. The points of inflection of the triphasic curve and the absolute values of recovery time, but not the slopes of the curves, are different in the two species of Halacaridae. However, the triphasic curve is well defined only for the experiments at room temperature. The 10-times larger species of *Halacarus* is more susceptible to the effects of asphyxiation than the species of *Rhombognathides*.

The relationship between time of anaerobiosis and time of recovery in the nematode *Enoplus communis* is an exponential one.

Kalmus (1942), in similar experiments, reported the recovery time to be different in different species and even mutants of *Drosophila*. In this genus, as in the halacarids over a certain period, a linear relationship between time of anaerobiosis and time of recovery was observed.

The second group of species, which consists of animals obviously able to leave and to repopulate the sea weeds at will, does not have to be adjusted for survival of anaerobic periods. The efficiency of receptor and locomotor organs of amphipods and related forms can always be considered as being sufficient to keep the animals in oxygenated water all the time. Accordingly *C. laeviusculus* has almost no resistance to the reduction of oxygen in its environment. The data in Figure 3 show that even five minutes of anaerobic conditions bring about the death of all members of this species, but the other two species of amphipods show some resistance. A related species of *Gammarus, G. duebeni,* is even known to occur on European coasts in rock pools and stagnant waters smelling of H_2S (Kinne, 1959).

The data of Figure 1 show that the least resistant amphipod, *C. laeviusculus,* has twice the oxygen consumption of the other two amphipod species. This might reflect the fact that the former species also leads a pelagic life, particularly in winter (Kunkel, 1918).

In a very general way it can be concluded that the nematode and the two halacarids in an anaerobic environment produce and deposit metabolic breakdown products which are toxic and cause paralysis. Up to a certain time, however, the tissues are not irreversibly injured by these breakdown products. The failure of *Calliopius laeviusculus* to endure even very short periods of de-oxygenation might be due either to its inability to gain sufficient energy under anaerobic conditions, or to the irreversibly harmful effects of even very small amounts of the breakdown products of anaerobic metabolism. The behavior of the remaining two amphipod species could be explained by assuming that either their energy or their excretory mechanisms function imperfectly under anaerobic conditions.

SUMMARY

1. The water trapped by large brown sea weeds at low tide may become oxygen-free in the night or on cloudy days.

2. Slow-moving, non-swimming animals living in the sea weeds all the time (like nematodes and mites) are paralyzed by the removal of oxygen from the water, but they recover from this state if the period of anaerobic stress has lasted less than approximately 16 hours at 25° C. The relationship between time of anaerobiosis and time of recovery is temperature-dependent and a characteristic of each of the three species investigated (*Enoplus communis, Rhombognathides seahami, Halacarus basteri basteri*).

3. Agile animals, capable of leaving and repopulating the sea weeds with the tides, show a different reaction pattern. One of the amphipods investigated (*Calliopius laeviusculus*) did not survive even a few minutes of anaerobiosis, while the other two species (*Gammarus oceanicus, Hyale prevosti*) survived from about half an hour to three hours of anaerobiosis. The former species occasionally leads a pelagic life and has twice the respiratory rate of the latter two species.

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ROLE OF LIGHT IN THE PROGRESSIVE PHASE OF THE PHOTOPERIODIC RESPONSES OF MIGRATORY BIRDS [1]

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Two conspicuous changes in physiological state precede the initiation of northward migration in late March in the slate-colored junco (*Junco hyemalis*)—gonadal recrudescence and fat deposition. The occurrence of these changes is regulated by day-length and in two separate phases. The first phase, called the preparatory phase, occurs in the fall and requires short days for its completion. The second phase, called the progressive phase, occurs in late fall and winter, and the rate at which it proceeds is a function of the daily photoperiod and is extremely rapid under long days. The summation of different degrees of physiological response to the daily light-dark cycles has been postulated to explain the events in these phases. (See Wolfson, 1959a, 1959b for review and references.)

The observations that interruption of the long night of a short day with a brief period of light during the progressive phase results in a rate of response comparable to that of a long day (Kirkpatrick and Leopold, 1952; Jenner and Engels, 1952) and that administration of the same total photoperiod in smaller doses induces a more rapid response (Wolfson, 1953), raised the question of the roles of light and darkness. The experiments reported here are concerned with the progressive phase and are part of an extensive series which was designed to determine the roles of light and darkness in both the progressive and preparatory phases. A brief summary of the results of all previous experiments and a preliminary report of one aspect of the present experiments have been reported (Wolfson 1959a, 1959b, 1959c).

The roles of light and darkness were explored in the present study by combining effective daily photoperiods with inhibitory dark periods and testing their effects in the winter and spring.

Materials and Methods

The species used was the slate-colored junco. Its geographic distribution and migration and the sequence of events in its annual cycle have already been described (Wolfson, 1952a). The birds were trapped during the period of fall and spring migration and were held in captivity under natural day-lengths (including civil twilight) until the experiment began. Artificial illumination only was used in all experimental rooms and was provided by 40-watt white fluorescent bulbs. Intensity varied from approximately 15 to 60 foot candles, depending on the dis-

[1] This work was supported by the National Science Foundation and the Graduate School of Northwestern University. It is a pleasure to acknowledge the able assistance of Betty Annan, Armin Sadoff, and David Winchester. I am also indebted to David Calhoun for a stimulating discussion of the results and for suggestions on analysis of the data.

tance of the cages from the light source. Temperature was not controlled and the rooms were unheated. In the winter experiment, the temperature ranged from 67°–79° F.; in the spring experiment it ranged from 60°–80° F. and was usually in the low 70's. Conditions of captivity, feeding, and care were similar to those already reported. Observations were made on fat deposition, body weight, and reproductive activity using methods described previously (Wolfson 1952a, 1954).

Experiments and Results

Experiment 1. In this experiment a stimulatory photoperiod, 12 hours, was combined with an "inhibitory" dark period, 16 hours, to give a 28-hour cycle. The experiment began on December 11 with 19 juncos and was continued to

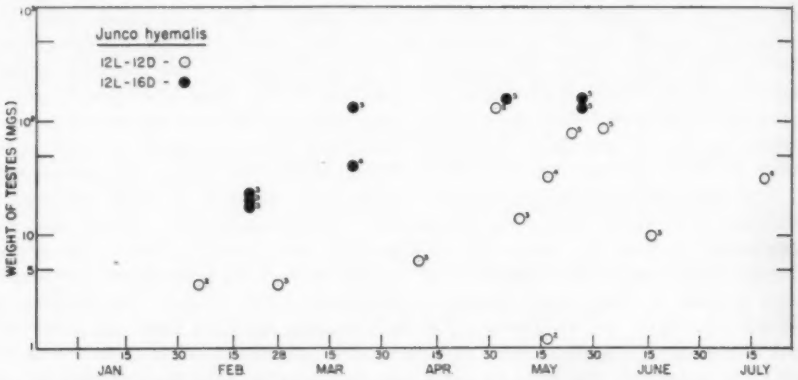

Figure 1. Testicular response in the light-dark cycles indicated. Weight of testes on logarithmic scale. Numbers adjacent to points indicate stage of spermatogenesis; stage 1 is minimum and 5 is maximum.

May 27, well beyond the normal time of response to experimental treatment, in order to determine the duration of the response, which is diagnostic for a 12-hour photoperiod. By the starting date, the birds had completed the preparatory phase.

Sixteen (11 ♂♂, 5 ♀♀) of the 19 birds showed a gonadal response. Figure 1 shows the rate of response for the responsive males and permits comparison of this rate with that of juncos treated with 12L–12D cycles beginning December 4 (Winn, 1950). The rate of response was clearly greater in the present experiment. The duration of response, however, was similar and was longer than that in birds treated with approximately 16-hour photoperiods, in which testicular activity ends by March 15 (Wolfson 1959b). The seminal glomera (vesicles) were not well developed, not even in the three birds autopsied in May which showed complete spermatogenesis (Stage 5). Histological study revealed normal spermatogenesis except perhaps in two males (autopsied on May 27) in which the germinal epithelium was extremely low and the lumens of the tubules extremely large. The mean ovarian weight for four responsive females autopsied in April and May was 26.3 (21.0–36.2) grams.

Seventeen of the 19 birds also showed a typical vernal fat response and concomitant increase in body weight. Figure 2 shows the mean body weights for the females and two groups of males, one of which responded sooner. The mean increase in weight (and range) from initial weight to maximum response weight (when birds were in the heavy fat class) was 5.2 (2.7–6.6) grams for 12 males and 5.4 (4.7–6.1) grams for 5 females. For the three groups graphed in Figure 2, the mean increases for each were, respectively, 5.6, 5.6, and 5.4 grams, and each represented a 31 per cent increase from the initial weight. Using the median date between successive weighings when the birds first showed moderate or heavy fat deposits, it took from 15–83 days for the fat response; the mean for the central

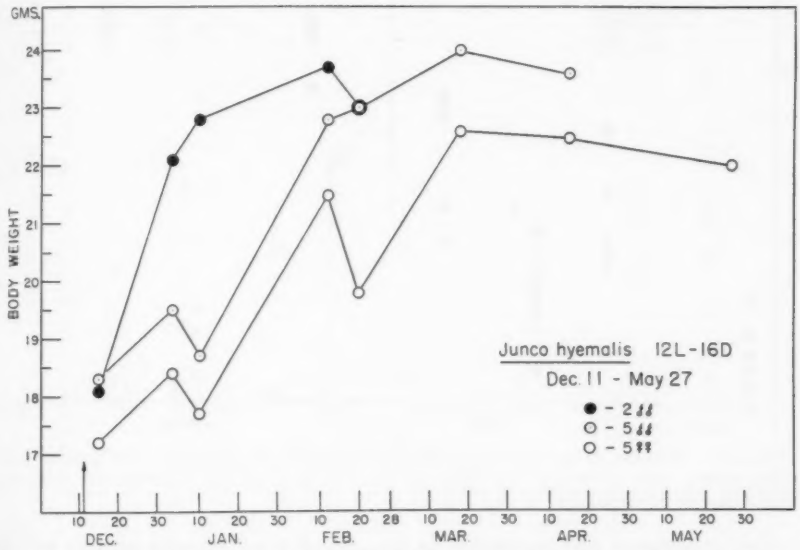

FIGURE 2. Changes in body weight in the 12L–16D group from December 11 to May 27. Each point is the mean weight for the three groups shown in the legend.

group of seven males was 54 days; for a similar group of four females it was 48 days. Comparable figures from the previous 12L–12D experiments (two separate experiments, and without separation of data for each sex) were 61 days and 79 days, and in these experiments only two males in a group of 23 responded in less than 55 days. The mean increase in weight for 12 birds in one experiment was 5.6 grams, which is almost identical with that in the present experiment.

Experiment 2. From the results of experiment 1 it seemed likely that the photoperiod determined the response and that the dark period was not inhibitory. But a possible weakness in that experiment was the ratio of light to darkness, which was close to one, and hence simulated a 12L–12D cycle. In an 8L–16D schedule, when the dark period appeared to be inhibitory, the ratio of dark to light was 2:1. To test further, therefore, the following schedules were selected,

using longer dark periods: 12L–20D; 16L–16D; 16L–22D; 16L–32D. The 16-hour photoperiod was selected because it is more stimulating than a 12-hour photoperiod; the 16L–32D cycle was chosen to obtain the same ratio of dark to light as in the nonstimulatory 8L–16D cycle. The lights were controlled automatically by time switches prepared specially for this study by the Aemco Corporation (Mankato, Minnesota).[2]

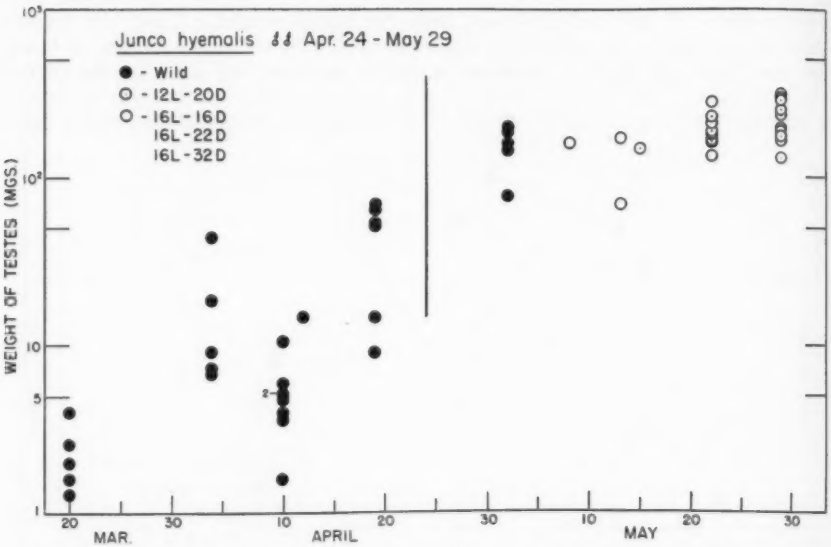

FIGURE 3. Weights of testes in wild and experimental birds in spring. Weights of testes on logarithmic scale. Vertical line indicates time of initiation of experimental light-dark cycles indicated in the legend.

This experiment was performed in the spring when gonadal growth and fat deposition were already underway, but continuation and maintenance of these responses are also regulated by photoperiod. For example, treatment with long daily dark periods, as in a 9L–15D cycle, beginning in late April, results in gonadal regression and loss of fat deposits within a few weeks; in cycles of 12L–12D, 20L–4D, and under natural day-lengths the responses continue, with a slower rate and a protracted response under 12-hour photoperiods.

The experiment began on April 24 and ended May 29; 68 juncos were used. The number and sex of the birds in each group were as follows: 12L–20D—11 ♂♂, 10 ♀♀; 16L–16D—3 ♂♂, 8 ♀♀; 16L–22D—6 ♂♂, 11 ♀♀; 16L–32D—2 ♂♂, 12 ♀♀.

Gonadal data were obtained for 22 ♂♂ and 41 ♀♀. The response of the males is shown in Figure 3, that of the females in Figure 4. Also shown are gonadal

[2] In a later study it was discovered that the light period in the 16L–32D cycle was 20–25 minutes shorter and the dark period correspondingly longer. This difference does not affect the design or results of the experiments, and for convenience the cycle is still referred to as 16L–32D.

data from wild birds and birds held under natural day-lengths to indicate the normal rate of growth and the condition of the gonads when the experiment began. It is clear from Figure 3 that all of the males maintained their reproductive activity. Histological study showed that spermatogenesis was normal except perhaps in a few cases where the lumens of the tubules were larger than normal because of the decreased depth of the germinal epithelium. There was a slight retardation in spermatogenesis in three birds, since they attained only stage 4 by the end of May. One occurred in each of the groups except the 16L–16D. The

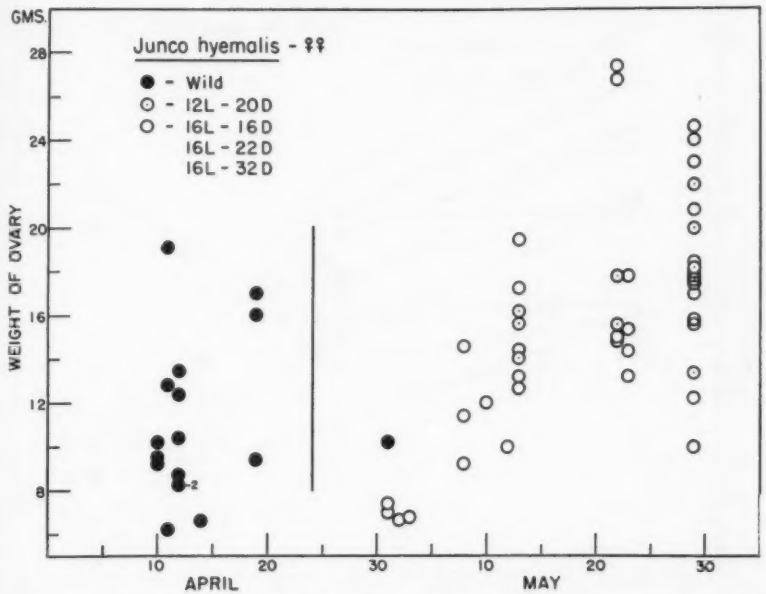

FIGURE 4. Weights of ovaries in wild and experimental birds in spring. Vertical line indicates time of initiation of experimental light-dark cycles.

seminal glomera were at maximal or submaximal size by May 29, except in a few cases. The testes of birds which received only 12 hours of light daily were as well developed as those in birds which received 16 hours of light.

The response of the females (Fig. 4) was more variable, but the results clearly indicate continuing growth of the ovary and maintenance of activity in all birds. The size of the largest follicles ranged from 1–2 millimeters by late May; this is the normal maximum size in captive birds. Inactive ovaries normally weigh less than 10 milligrams. The mean weight (and range) for four inactive ovaries obtained in the first 10 days of the experiment was 7.0 (6.6–7.4) milligrams.

Of the 68 birds in the experiment, 64 lost weight and reached below-normal weights which ranged from 12–15 grams. Only four birds failed to show abnormal losses in weight, and three of these were in the 16L–16D group. Represent-

ative data for individual birds are presented in Figure 5, which shows the weight changes in the males in the 12L–20D group. The individual curves show the uniformity of response. The weight loss, to about 18 grams, was due mostly to loss of fat from subcutaneous and intraperitoneal depots. The mean loss was about 6 grams and the percentage lost (from the initial mean weight) was about

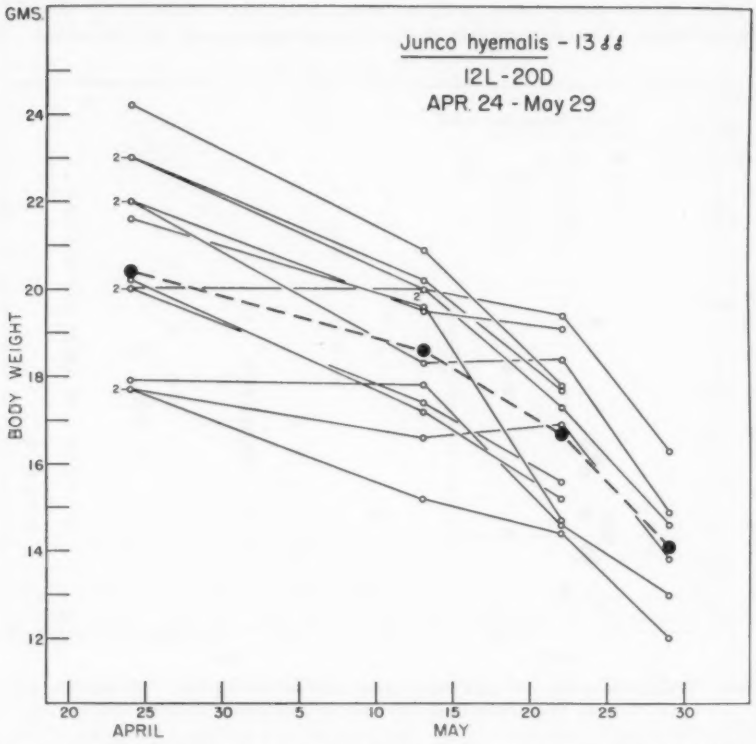

FIGURE 5. Changes in body weight in individual male juncos treated with 12L–20D cycles in spring. Mean for the group shown by large points and dashed line.

31 per cent. Adequate numbers of females occurred in all groups to permit calculation of the mean weight curves which are given in Figure 6. Marked losses occurred in all groups, reaching abnormally low levels especially in the 12L–20D and 16L–22D groups. The weight loss of 32 per cent in 12L–20D females was almost identical to that in the males.

It is evident that the gonadal response continued despite excessive losses in weight. For 12 birds that showed the greatest gonadal weights on May 22 and May 29, the mean weight loss was 5–6 grams except in the 16L–16D group where it was 2–3 grams. No. 464 in the 16L–22D group had the largest testes of all

the birds on May 29 (312.6 milligrams); its body weight was 13.4 grams, which was 6.6 grams less than its initial weight.

Discussion and Conclusions

From the results of their studies Kirkpatrick and Leopold (1952) and Jenner and Engels (1952) assigned an inhibitory role to the long dark period of short winter days. On the basis of the experiments reported here it seems unlikely

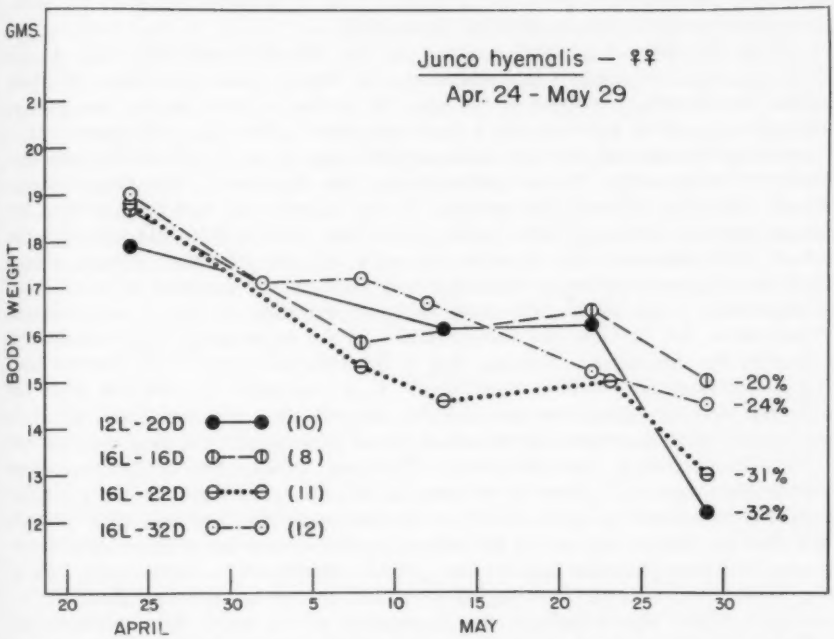

Figure 6. Changes in mean body weight in females in the light-dark cycles indicated. Number of birds in each group is given in parentheses in the legend. Percentage lost is based on mean initial weight.

that the dark period is inhibitory. The responses simulated those obtained with similar photoperiods rather than similar dark periods. This was brought out particularly by the 12L–16D schedule in the first experiment. Not only did the birds respond, but the duration of the response was longer than normal and characteristic of 12-hour photoperiods (Wolfson, 1952b, 1959b). Also, the annual molt was not induced and this also is characteristic of exposure to 12-hour photoperiods. By contrast, birds treated with approximately 16-hour or longer photoperiods beginning in December showed a shorter duration of response and were usually molting by April 1.

In the spring experiments, long dark periods also failed to inhibit gonadal

activity. It is clear that an effective photoperiod, for example, 12 or 16 hours long, can maintain gonadal activity despite long intervening dark periods, even when the dark period is twice as long as the light period, as in the 16L–32D schedule. The ability of the birds to maintain gonadal activity under this schedule raised the question of whether such a schedule could induce the gonadal response in winter. This was tested recently, and not only was a gonadal response obtained but also a fat response (Wolfson and Winchester, unpublished data). The occurrence of the fat response was completely unexpected, since the birds lost weight in the present experiment. Again, a long dark period failed to be inhibitory when coupled with an effective photoperiod.

When the rate of gonadal response in the 12L–12D and 12L–16D groups (Fig. 1) was compared, it was surprising to find a faster rate when the dark period was longer. Analysis of the time of the fat response in the two groups also showed a clear tendency for a faster response in the 12L–16D group. It is interesting that although the rate of the gonadal response in the 12L–16D group was similar to that under 16-hour photoperiods, the duration of the response was clearly that of a 12-hour photoperiod. In the experiment just cited, where the fat and gonadal responses were induced in winter with a 16L–32D schedule, the rate of both responses also exceeded that of a 16L–8D schedule. Hence, a long dark period, instead of being inhibitory, may augment the response to an effective photoperiod. This seems clear from the data available so far in two different experiments, but in view of other variables in the experiments being compared, especially the difference in starting time in the 16L–32D experiment, further testing is necessary and will be undertaken. It is reasonable to conclude from the data reported here that there is a relation between the photoperiod and the dark period, and that an effective photoperiod cannot be negated by a long dark period.

Earlier studies in our laboratory (Wolfson, 1952b) led to the hypothesis that in the progressive phase of the gonadal and fat cycles there are daily physiological increments of response which are a function of effective daily photoperiods, and that the rate of response is determined by the summation of these daily increments. Farner, Mewaldt and Irving (1953) postulated a carry-over effect of the photoperiod into the dark period to explain the effectiveness of short periods of light. They also stipulated the summation of the total daily photoperiodic effect. On the other hand, Kirkpatrick and Leopold (1952) and Jenner and Engels (1952) assigned an active regulatory role to the dark period. All of these interpretations have been derived indirectly from the manifested response of the "effectors"—the gonads and the fat depots. Very little is known about the dynamics of the specific events which regulate the gonadal and fat cycles, but two organs are clearly involved, the hypothalamus and the pituitary (see Wolfson, 1959b, and Farner, 1959, for review and summary). The results of the present experiments lend support to the primary role of the photoperiod, and there is no evidence for an inhibitory role of darkness. When there is no manifest response in a particular photoperiodic schedule, for example 8L–16D, the failure appears to be caused by an ineffective photoperiod, for when the photoperiod was increased to 12 hours a response occurred, even though the dark period remained the same. The difference in rate of response in the 12L–12D and 12L–16D groups suggests that the daily response may be a function of the photoperiod and the subsequent

dark period. The dark period could function as a period of time which permits the effect of the photoperiod to continue, as postulated in the carry-over effect, but this would not explain the difference in rate of response in the 12L–12D and 12L–16D groups if the duration of the carry-over effect is the same for a given photoperiod.

One of the questions which was asked in previous studies (Wolfson, 1959a) was whether the bird could "store" the effects of stimulatory light-dark cycles when nonstimulatory cycles intervened. Cycles of 8L–16D were alternated with 16L–8D cycles and the birds responded, but it was not possible to answer the question of storage because the birds could have been responding not to the different daily cycles but rather to the schedule as a whole, for example, 16L–16D–8L–8D, and from other studies it was known that 16L–16D and 8L–8D are stimulatory cycles. The question of storage is perhaps answered by the results of the present experiments. In the 16L–32D cycle, to use an extreme example, the effect of the light period was not negated by the extended dark period. Or, if the bird has a 24-hour rhythm and responded to the 16L–32D cycle on that basis, then it would have experienced alternating days of 16L–8D and 24D. In any case, if gonadotropins are released during effective photoperiods, the testes appear to respond to them each day, or in each cycle, and long intervening dark periods do not inhibit this reaction. Hence, the effects of stimulatory photoperiods appear to be stored each day and summated to the point where the gonadal and fat responses are manifested.

Summary

1. To determine the roles of light and darkness in the photoperiodic responses of birds, effective photoperiods were combined with normally inhibitory dark periods to give cycles longer than 24 hours as follows: 12L–16D; 12L–20D; 16L–16D; 16L–22D; 16L–32D. The experiments were performed in winter and spring, and tested both induction and maintenance of the gonadal and fat responses.

2. The results indicate that the photoperiod and not the dark period determined the response. Long dark periods *per se,* therefore, are not inhibitory.

3. Comparison of the rate of response in previous experiments with similar photoperiods but different dark periods shows a greater rate of response with longer dark periods. The response to a given light-dark cycle may, therefore, be a function of the photoperiod and the subsequent dark period.

4. The results also indicate that the gonadotropic and lipogenetic effects of each stimulatory light-dark cycle are "stored," or cannot be negated by long intervening dark periods.

5. The relation of these findings to theories of the mechanism of response to light and dark is discussed.

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AN ARGINASE INHIBITOR(S) AND ITS POSSIBLE ROLE IN THE DEVELOPMENTAL DECREASE OF ARGINASE ACTIVITY IN CHICK EMBRYOS[1]

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A number of enzyme activities have been measured in crude homogenates of developing embryos, and in several instances changes in activity have been correlated with morphological events and the time tissues and organs begin to function: *e.g.* cholinesterase in amphibian embryos (Sawyer, 1943). Such correlations suggest that enzyme activities measured in crude homogenates reflect events involved in metabolic differentiation. Consequently, it seems reasonable to believe that studies of mechanisms by which enzyme activities change will also yield information concerning mechanisms involved in differentiation at the biochemical level. With this assumption in mind we began studying arginase activity in developing chick embryos in an attempt to establish the cause of the decrease in activity per unit moist weight which occurs during development (Needham and Brachet, 1935; Fisher and Eakin, 1957; Clark and Fischer, 1957; Roeder, 1957).

At the outset of these studies we assumed that the arginase activity expressed by crude homogenates is primarily a measure of the amount of enzyme present. Evidence obtained in the course of surveying activity in various tissues led us to question this assumption. Subsequently, we observed that several tissue homogenates which exhibit little or no arginase activity are capable of inhibiting the activity of embryo homogenates. Preliminary studies indicate that inhibition is due to the presence of a high molecular weight inhibitor(s) and are consistent with the possibility that it is ribonucleic acid in nature. The presence of an arginase inhibitor(s) in chick tissues raises the possibility that the developmental decrease in activity could be due to an increase in the concentration of this agent as well as a decrease in the concentration of enzyme.

Materials and Methods

Eggs from a flock of Rhode Island Reds were incubated at 39° C. in a commercial incubator. Eggs were removed at various times, cracked in a pan of water and the entire living area excised. The vitelline membrane was removed and embryo, pellucida, vasculosa and vitellina were separated. At the 24-hour stage no vascular area is present; however, a pre-vascular area exists, containing mesodermal tissue (Hamilton, 1952) which we refer to as vascular area. At several ages, area vitellina and area vasculosa were separated into two layers: ectoderm and endoderm in the case of vitellina, somatopleure and splanchnopleure in the case of vasculosa. The various tissues were weighed and homogenized in distilled

[1] Supported by a grant from the United States Public Health Service, National Institutes of Health (Grant No. R6-4667).

water in the cold with a pestle homogenizer having a teflon pestle and a glass vessel. Homogenates were assayed for arginase activity using the procedure described below.

Enzyme was activated by heating 4 ml. of homogenate with 0.2 ml. of a 20 per cent solution of $MnCl_2 \cdot 4H_2O$ for 20 minutes at 57° C. Activated homogenate was equilibrated to 30° C. in a water bath and 1 ml. of an 18 per cent solution of arginine hydrochloride at pH 9.5 was added. A saturated solution of NaOH was used to adjust the pH of the arginine solution. One-ml. samples of reaction mixture were removed at 0, 20, 40 and 60 minutes after arginine addition. The samples were heated to 90° C. for 10 minutes to stop enzymatic activity. Two and one half ml. of 0.2 *M* phosphate buffer at pH 6.8 were added to each sample

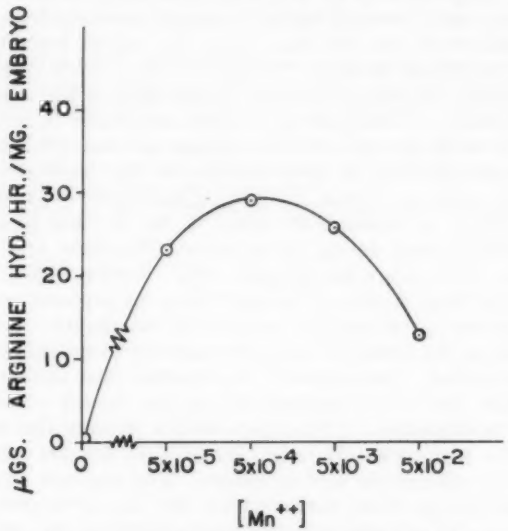

FIGURE 1. Effect of Mn^{++} concentration on arginase activity of 4-day embryo homogenate (15 mg. per ml. reaction mixture).

and 0.2 ml. of a glycerol urease preparation from jack bean meal (Folin and Wu, 1919) was used to hydrolyze urea present. After incubation with urease for 10 minutes at room temperature, 2 ml. of half-saturated Na_2CO_3 were added and the ammonia transferred to 0.05 N H_2SO_4 by a stream of ammonia-free air. Nessler's reagent (Folin and Wu, 1919) was added to the acid solutions and the color read on a Klett-Summerson colorimeter, using filter number 54. Ammonia nitrogen present was calculated from a standard curve prepared with analytical grade ammonium sulfate. Nitrogen values for the sample inactivated immediately after arginine addition were subtracted from all values and the corrected values were plotted against time of incubation with arginine. The slope of the resulting curve was used as a measure of arginase activity. In this report activities are presented as μg. arginine hydrolyzed per hour per ml. reaction mixture or per mg. tissue.

Activation of arginase

The procedure outlined above gives a 5×10^{-2} *M* concentration of Mn^{++} in the activation mixture. Van Slyke and Archibald (1946) and Greenberg (1955) recommend this concentration to activate liver arginase. We have found that this concentration gives full activity for some but not all chick embryo tissues, *e.g.* 4-day embryo homogenates give maximum activity with 5×10^{-4} *M* Mn^{++} (Fig. 1). In surveying activity changes during development and in experiments involving tissue mixtures, it seemed desirable to use the same concentration throughout. Consequently, 5×10^{-2} *M* Mn^{++} was almost always used. In some experiments involving 4-day embryos 5×10^{-4} *M* Mn^{++} was used. These cases will be noted in the text. Also, $MnSO_4$ in maleate buffer (Greenberg, 1955) was used to activate and found to give the same result as $MnCl_2$ in distilled water.

Van Slyke and Archibald (1946) recommend heating to 57° C. for 20 minutes in the presence of Mn^{++} to activate arginase in crude liver homogenates. Arginase appears to be stable under these conditions. We have found that partially purified liver arginase gives the same activity whether heated or not. Greenberg (1955) recommends heating to 60° C. for 20 minutes as one step in arginase isolation. It should be noted that heating in the absence of Mn^{++} destroys all activity of homogenates (Fig. 1).

Incubation with substrate

The procedure outlined above gives a final arginine concentration of 0.17 *M* in the reaction mixture. A study has been made of 4-day embryo arginase activity at various substrate concentrations. These results give an apparent K_m of 8×10^{-3} *M* which is in good agreement with values of 11.6 and 7.7×10^{-3} reported for liver arginase (Greenberg, 1951).

Most tissues have been assayed at two or more concentrations and in all cases activity appears to be linear with concentration (Figure 5 contains data for 4-day embryo activated at 5×10^{-4} *M* Mn^{++}).

The pH of reaction mixtures prepared as described above is 9.2. Activities have been measured at several hydrogen ion concentrations and maximum activity was found at pH 10, which is in agreement with results reported by Greenberg (1951).

Measurement of urea formed

Phosphate buffer addition to heated samples as described previously gives a pH of 7.5. Urea present has been shown to be quantitatively hydrolyzed by treatment with urease under these conditions. Added urea can be recovered quantitatively from reaction mixtures as ammonia ($\pm$ 2 μg. N per ml. reaction mixture). When urease is omitted from the procedure, no arginase activity is observed. Arginase activities can be duplicated on the same homogenate, $\pm$ 2 μgs. urea nitrogen or $\pm$ 12 μg. arginine hydrolyzed per hour per ml. reaction mixture. When no arginase activity was observed, a value was calculated consistent with the sensitivity of the assay (12 μg. arginine hydrolyzed per hour per ml. reaction mixture).

Results

Distribution of arginase activity in developing eggs

The first objective of this study was to describe the changes in arginase activity of several tissues in eggs during the first 7 days of development. Embryo, pellucida, vasculosa and vitellina were excised, weighed and homogenized. An aliquot was removed for dry weight determination. Before the living area was dissected, the distance from the center to the outer edge of pellucida, vasculosa and vitellina was measured (several measurements were made on each tissue in each egg and the averages recorded). From these studies we could follow changes in the moist weight, dry weight, surface area and arginase activity per unit moist weight, dry

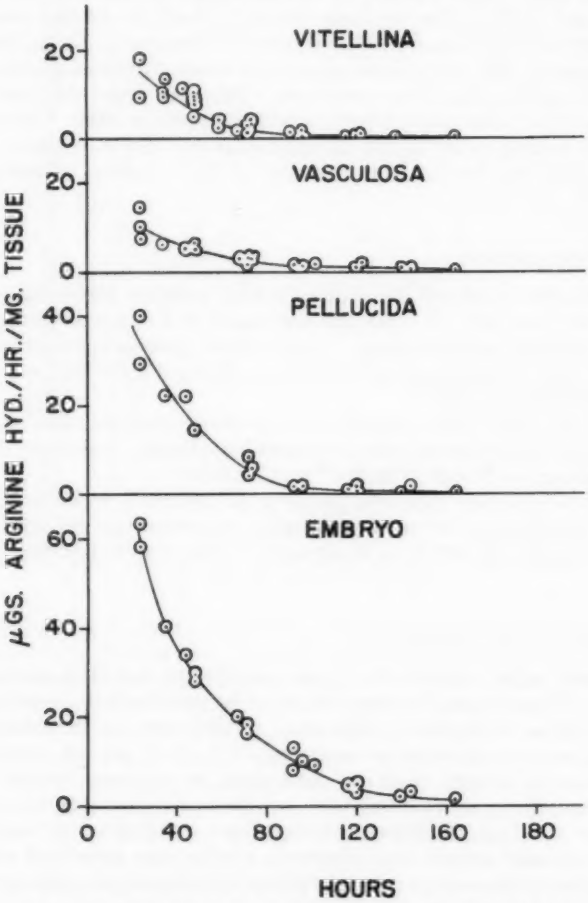

Figure 2. Arginase activities of various tissues in developing eggs.

TABLE I

Arginase activity of tissue layers in yolk sac of developing eggs

Incubation time of eggs—hours	Mg. ectoderm per mg. vitellina	μg. arginine hydrolyzed per hour per mg. tissue			
		Ectoderm	Endoderm	Vitellina	Calculated activity of vitellina
64	0.38	8.2	1.2	1.3	3.9
73	0.37	9.8	2.0	2.6	4.9
65	0.35	13.9	0.0 (<0.9)	1.8	4.8
	Mixture of homogenates—35% ectoderm			1.8	4.8
98	0.33	4.5	0.0 (<2.0)	1.7	1.5
	Mg. somatopleure per mg. vasculosa	Somatopleure	Splanchnopleure	Vasculosa	Calculated activity of vasculosa
73	0.25	8.7	0.0 (<0.5)	1.7	2.2
144	—	1.0	0.0 (<0.3)	0.0 (<0.2)	—

weight and surface area. Embryos increased in moist weight in a pattern almost identical with that reported by Schmalhausen (1926). Pellucida increased in moist weight from 2 mg. at the first day to 300 mg. on the seventh day, vasculosa increased from 10 to 800 mg. and vitellina increased from 46 on the first day to 300 on the third day and subsequently decreased to 180 mg. on the seventh day. Surface areas of pellucida, vasculosa and vitellina followed essentially the same pattern as moist weight, *i.e.*, pellucida and vasculosa increased in area from the first to the seventh day and vitellina increased to a maximum on the third day. An aliquot of each homogenate was dried at 60° C. for two days in a petri dish and the residue weighed. Per cent dry weight was essentially constant in each tissue during the first 7 days of development (embryo 9 per cent, pellucida 10 per cent, vasculosa 17 per cent and vitellina 13 per cent). Arginase activity decreased during development in each tissue when calculated per unit moist weight, dry weight or surface area. Activities calculated per unit moist weight are shown in Figure 2. When activity at the first day was assigned a value of 100 per cent for each tissue, it was found that the pattern of change during incubation was almost the same in all tissues. Area vitellina is of particular interest because of the way it spreads over the surface of yolk. Apparently (Hamilton, 1952), this tissue spreads by addition of cells to its outer edge, which are formed from periblastic syncytium at the zone of junction. If this picture is correct, tissue at the outer edge is younger, relative to the time cells are formed, than in the inner area. We measured arginase activity in a strip of tissue approximately 10 mm. wide around the outer edge of vitellina. From the first to the seventh day this strip exhibits substantially the same activity as the remainder of vitellina. It follows that the mechanisms which cause activity to decrease are operating in the non-cellular syncytium which becomes vitellina; otherwise, activity in the peripheral strip should not decrease as rapidly as in the remainder of vitellina. These observations suggest that all tissues in developing eggs tend to decrease in arginase activity and that the activity decrease is not correlated with any obvious developmental event.

Data in Figure 2 show that tissues exhibit a common tendency to decrease in arginase activity; however, these tissues show marked differences in their level of activity whether calculated on a moist weight or dry weight basis. It appears that there is a gradient of activity related to difference in proximity to yolk. Embryo, the most distant, exhibits the highest arginase activity; pellucida the next most distant, exhibits the next highest activity; and yolk sac tissues in contact with yolk exhibit the lowest activity. Results in Table I show a very significant difference in activity between ectoderm and endoderm of vitellina, and between somatopleure and splanchnopleure of vasculosa. In every case the tissue in contact with yolk material exhibited a much lower activity than the tissue distant from yolk. Consequently, the gradient can be extended to the tissue layers of yolk sac. Furthermore, it was possible to calculate the activity of total tissue (vitellina or vasculosa) from the weights and activities of its component layers (Table I). It was found that at approximately the third day of incubation there was a significant difference between the calculated activity of vitellina and the measured activity. In one case enough of the tissues were obtained so that homogenates of ectoderm and endoderm could be assayed together and separately. In this case endoderm homogenate clearly inhibited the activity of ectoderm. These results are consistent with the possibility that endoderm, at least at one stage, is capable of inhibiting the arginase activity of ectoderm.

Inhibition of embryo arginase activity

Results described in the preceding section suggested that endoderm of area vitellina contains an arginase inhibitor(s). Greenberg (1951) has reported that most amino acids inhibit arginase. It was conceivable that yolk sac tissues contain relatively large amounts of free amino acids from digestion of yolk proteins. An attempt was made to demonstrate inhibitor activity in endoderm using embryo homogenates as sources of arginase activity (Table II). Endoderm homogenates were dialyzed in an effort to remove possible low molecular weight inhibitors (Table II). Dialysis was carried out in the cold against distilled water. Results show that endoderm homogenates do inhibit but that dialysis does not remove inhibitory activity as would be expected if the agents were amino acids. Furthermore, casein hydrolysate, obtained commercially, does not inhibit at a dry weight concentration equal to that of endoderm which is inhibitory. From these results we concluded that inhibition of embryo arginase by endoderm homogenates is not due to free amino acids in the endoderm. It should be noted that dialysis actually increases the inhibitory activity of endoderm homogenates. An explanation of this effect will be presented in a later section of this report.

Preliminary studies of the nature of the inhibitor(s) have been undertaken, using vitellina of eggs incubated 4 days as a source of inhibitor. The dialysis effect described above has been confirmed with vitellina. An attempt was made to destroy inhibitory activity by heating to 90° C. for 20 minutes. Results obtained show that this treatment does not destroy inhibitory activity but actually increases inhibition. These results suggest that the inhibitor(s) is a high molecular weight, heat-stable substance. Moss (1952) reported that sodium ribonucleate from yeast inhibits arginase. We have confirmed this observation, using 4-day embryo as a source of arginase activity. The first two steps of a ribonucleic acid

(RNA) isolation procedure were applied to area vitellina homogenate (Kay and Dounce, 1953). Inhibitory activity was found in the fraction which, in the case of mammalian liver, contains RNA. Four-day vitellina was homogenized in a solution of NaCl and sodium citrate. The homogenate was centrifuged, yielding a supernatant and precipitate (ppt. 1). The supernatant was adjusted to pH 4.5 with HCl and centrifuged, yielding ppt. 2 and supernatant which was adjusted to pH 7.0 with NaOH for testing (final supernatant). The two precipitates were suspended in distilled water. These three fractions were assayed for arginase

TABLE II

Inhibition of embryo arginase by endoderm homogenates

Incubation time of eggs—hours	Mg. per ml. reaction mixture		μg. arginine hydrolyzed per hour per mg. embryo
	Embryo	Endoderm*	
70	5.4	0	14
	5.4	2	10
	5.4	3	8.0
	5.4	2 (dial. 1 day)	0 (<2)
	5.4	3 (dial. 1 day)	0 (<2)
96	15	0	7.9
	15	140	4.6
	15	140 (dial. 1 day)	0.8
	15	140 (dial. 4 days)	0 (<0.8)
	15	140 (dial. 7 days)	0 (<0.8)
140	40	0	3.9
	40	9	3.4
	40	12	2.8
	40	9 (dial. 1 day)	1.6
	40	12 (dial. 1 day)	1.2
161	175	0	0.90
	175	125	0.50

* Endoderm homogenates did not exhibit arginase activity alone at the concentrations used in any of these experiments.

activity and arginase inhibitor activity using 4-day embryo homogenate as a source of arginase activity. Only ppt. 1 exhibits arginase activity and only ppt. 2 inhibits embryo arginase. Mixtures of ppt. 1 and embryo in three assays yielded higher activity than predicted. This result can be explained by the presence of an arginase stimulatory agent in embryo. Evidence for the existence of such an agent will be presented in a later report.

These preliminary studies indicate that the inhibitor(s) is high molecular weight in nature and are consistent with the possibility that it is RNA.

Distribution of inhibitor

The presence of inhibitor(s) in tissues which do not exhibit arginase activity can be demonstrated by showing that homogenates inhibit the arginase activity of

4-day embryos, as was done with area vitellina and area vitellina endoderm. It was found that heart from 21-day embryos does not exhibit arginase activity and inhibits 4-day embryo arginase (Fig. 3). As in the case of vitellina and vitellina endoderm, dialysis for 4 days in the cold against distilled water increases the inhibitory activity of heart homogenate (Fig. 3). Heart from 6-week-old chickens also inhibits 4-day embryo arginase. Results obtained show this inhibition when enzyme is activated with 5×10^{-2} or 5×10^{-4} M Mn^{++}.

In order to directly demonstrate the presence of inhibitor(s) in a tissue which exhibits arginase activity, it is necessary to destroy its arginase activity before testing against 4-day embryo arginase. It is known that dialysis inactivates

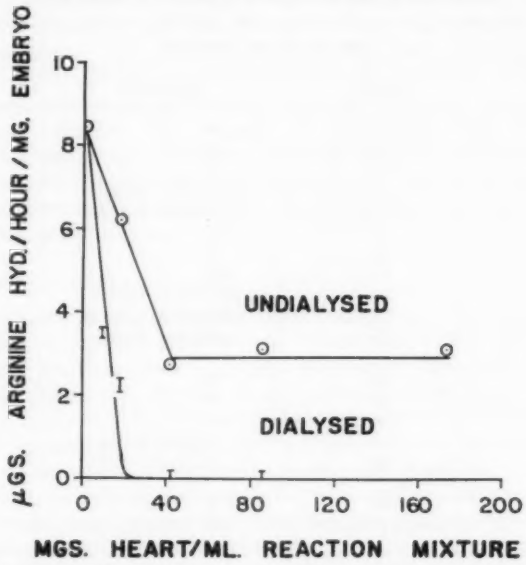

FIGURE 3. Effect of 21-day embryo heart homogenate on the arginase activity of 4-day embryo (15 mg. per ml. reaction mixture). Dialysis was carried out for 4 days in the cold against distilled water.

arginase (Greenberg, 1951). We have confirmed this observation, using partially purified beef liver arginase which was obtained commercially. Activation with Mn^{++} under the conditions we are using does not restore activity. We have destroyed the arginase activity of several tissue homogenates in this manner and tested the dialyzed homogenates for inhibitor activity, using 4-day embryos as a source of arginase activity. It was observed that homogenates of bone marrow, skin, leg muscle, eye and brain from 21-day embryos can completely inhibit the arginase activity of 4-day embryos. The effect of dialyzed brain homogenate is shown in Figure 4. These results clearly show that all tissues tested contain arginase inhibitor(s), if the only effect of dialysis is to inactivate arginase. The observation was made with vitellina, vitellina endoderm and heart that dialysis

increases the inhibitor activity of tissue homogenates which express little or no arginase activity. This is inconsistent with the above interpretation unless these tissues do contain arginase which does not exhibit activity because of the presence of inhibitor. Evidence is presented in the following section that heart does contain arginase even though crude homogenates do not exhibit arginase activity. Earlier it was shown that heating vitellina homogenate to 90° C. for 20 minutes, which inactivates arginase, also increases inhibitor activity. These results are consistent with the interpretation that the primary effect of dialysis is to inactivate arginase.

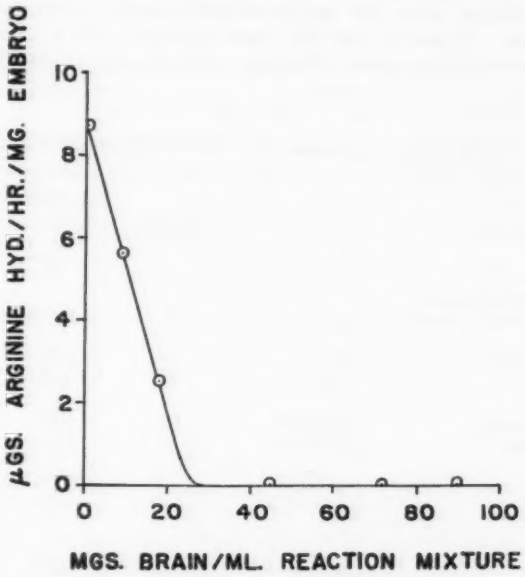

FIGURE 4. Effect of dialyzed brain (from 21-day embryos) homogenate on the arginase activity of 4-day embryo (15 mg. per ml. reaction mixture). Dialysis was carried out for 4 days in the cold against distilled water.

Role of inhibitor in establishing the arginase activity of crude homogenates

If inhibitor(s) is playing a major role in establishing the arginase activity expressed by homogenates, it is necessary that inhibitor(s) be effective against enzyme in the same tissue as well as against 4-day embryo arginase. Homogenate of heart from 6-week-old chickens has been shown to inhibit 4-day embryo arginase. It was found that 8-day embryo heart exhibits arginase activity and that heart from 6-week-old chickens inhibits this activity. A second type of evidence is that dialyzed 4-day embryo homogenate inhibits the arginase activity of undialyzed 4-day embryo homogenate. From these results we believe it is reasonable to assume that inhibitor(s) is effective against enzyme in the same tissue as well as 4-day embryo arginase.

If inhibitor(s) is playing a major role in establishing the activity of crude homogenates, it is necessary that tissues containing inhibitor(s) contain more enzyme than is indicated by the activity of crude homogenates. We attempted to test this possibility by partially isolating arginase from heart of 6-week-old chickens, which does not exhibit arginase activity as a crude homogenate. The arginase isolation procedure recommended by Greenberg (1955) for horse liver was applied to heart (Table III). These results show that heart contains at least 30-fold more enzyme than is indicated by the activity of crude homogenate. It appears that acetone precipitation "unmasks" arginase activity, presumably by inactivating inhibitor since the acetone-soluble fraction does not inhibit 4-day embryo arginase. A similar case has been reported with a pantothenic acid-requiring mutant of Neurospora (Wagner and Guirard, 1948; Wagner, 1949).

TABLE III

Partial isolation of arginase from hearts of 6-week-old chickens

Isolation step	Fraction	Per cent original N	μg. arginine hydrolyzed per hour per	
			Mg. original tissue	Mg. N in fraction
Heart homogenate incubated overnight with Mn^{++} at pH 7.6	extract A	100	0 (<0.02)	0 (<3)
Acetone precipitation	soluble	6	0.03	81
	insoluble	84	0.62	118
Extraction of acetone Powder with buffer	insoluble	66	0.15	37
	extract B	11	0.26	370
Extract B heated at 60° C. for 20 minutes	precipitate	3	0 (<0.01)	0 (<31)
	extract C	5	0.24	810
Extract C dialyzed and lyophilized	powder D	1.4	0.19	2180

Non-growing pads and homogenates of wild type are capable of coupling pantoyl lactone and beta-alanine to form pantothenic acid. Such preparations of the mutant do not show this activity; however, acetone powders of both organisms can catalyze the reaction. As in the case of arginase, acetone precipitation "unmasks" an enzyme activity.

Possible role of inhibitor(s) in the developmental decrease of arginase activity

Evidence has been presented in a previous section that area vitellina contains an arginase inhibitor(s) and that arginase activity in this tissue decreases during development in a manner similar to other tissues, *e.g.* embryo. Inhibitor activity has been demonstrated in all tissues tested. Results presented in the previous section indicate that inhibitor(s) is playing a role in establishing the activity of crude homogenates. Consequently, the developmental decrease of activity could

be the result of an increase in the concentration of inhibitor(s), a decrease in the concentration of enzyme, or the concentration of both agents could change, the net result being a decrease in arginase activity. Even if the concentration of inhibitor remains constant, its presence would cause activity to decrease more than would be warranted by a decrease in enzyme concentration, since there would be an increase in the inhibitor(s)-to-enzyme ratio. The developmental decrease in arginase activity would accurately represent a decrease in enzyme concentration only in the event that enzyme exhibits a *constant* fraction of its activity in various tissues during the developmental period studied. If the modifying influence of homoge-

TABLE IV

Arginase activity of mixtures of 21-day embryo tissues with 4-day embryo

Tissue	Mg. per ml. reaction mixture		μg. arginine hydrolyzed per hour per		
			Ml. reaction mixture		Mg. tissue
	Tissue	Embryo	Experimental	Calculated	
Brain	0	20	180		9.0
	90	20	118	298	
	90	0	118		1.3
Leg muscle	0	10	81		8.1
	82	10	69	150	
	82	0	69		0.84
Eye	0	20	149		7.5
	45	20	193	286	
	45	0	137		3.0
Bone marrow	0	10	69		6.9
	36	10	69	88	
	36	0	19		0.53
Skin	0	10	56		5.6
	63	10	124	125	
	63	0	69		1.1

nates on the activity of arginase is constant in various tissues, mixtures of tissues should exhibit an activity equal to the sum of the activities of the tissues measured separately. Homogenates of several tissues from 21-day embryos were mixed with homogenate of 4-day embryo and the mixtures assayed for arginase activity (Table IV). From these results we concluded that mixtures of tissue homogenates do not necessarily exhibit an activity equal to the sum of the activities of the tissues measured separately. Only in the case of skin was activity equal to the calculated value. Apparently, embryo does not exhibit any activity in the presence of brain or leg muscle. These results are not consistent with the possibility that the modifying influence of all homogenates is the same. Consequently, we believe that the different arginase activities expressed by various tissue homogenates can not be explained simply by differences in enzyme content.

Results described in Table IV can be explained with the assumption that tissue homogenates are effectively mixtures of enzyme and inhibitor(s), provided the enzyme-inhibitor interaction is reversible. Evidence for reversibility has been obtained, using 4-day embryo as a source of arginase activity and heart from 6-week-old chickens to inhibit. Results presented in Figure 5 show that this system exhibits the properties which, according to Ackermann and Potter (1949), are characteristic of reversible inhibition. If tissue homogenates are effectively mixtures of enzyme and a reversible inhibitor(s), mixtures should exhibit activities equal to or less than the sum of the components but equal to or greater than the

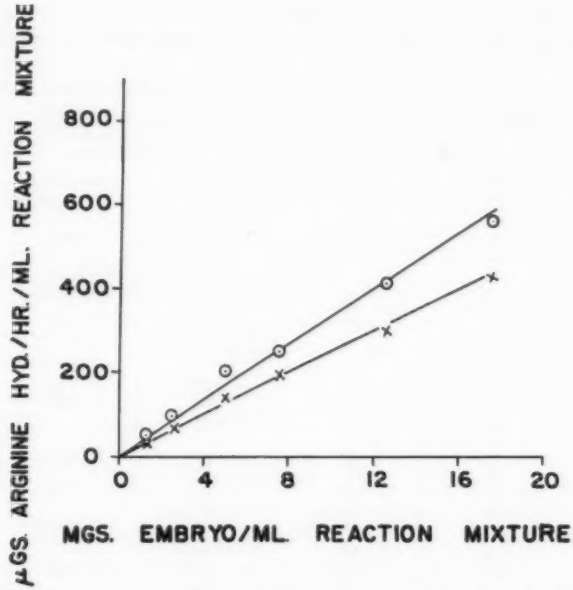

FIGURE 5. Effect of heart from 6-week-old chickens (120 mg. per ml. reaction mixture) on the arginase activity of 4-day embryo at various concentrations. Activated with 5×10^{-4} *M* Mn^{++}. ⊙ embryo alone, X plus heart.

activities of the least active components. Results in Table IV are consistent with these requirements. If the activity of a tissue homogenate is determined by the presence of a reversible inhibitor as well as enzyme, it is possible that activity would not be proportional to tissue concentration. Data in Figure 5 show that the arginase activity of 4-day embryos is proportional to tissue concentration over a 10-fold range. Such a proportionality has been shown by Straus and Goldstein (1943) when the ratio of inhibitor concentration to dissociation constant is greater than 100 (zone c). Our results can be explained on the basis of zone c inhibition.

Assuming that homogenates are effectively mixtures of enzyme and inhibitor(s), the data in Table IV require that different tissues have different inhibitor(s)-

to-enzyme ratios. Consequently, enzyme does not exhibit the same fractional activity in all tissues and, therefore, inhibitor(s) must be playing some role in the differentiation process which results in each tissue having its characteristic arginase activity, as measured in homogenates. This suggests that the developmental decrease in activity could also be at least in part due to a change in inhibitor concentration. We have found that heart arginase activity decreases between the eighth and the twenty-first day of incubation. At the twelfth day, heart exhibits a small but significant arginase activity and does not inhibit 4-day embryo arginase. At the twenty-first day, heart does not exhibit a measurable arginase

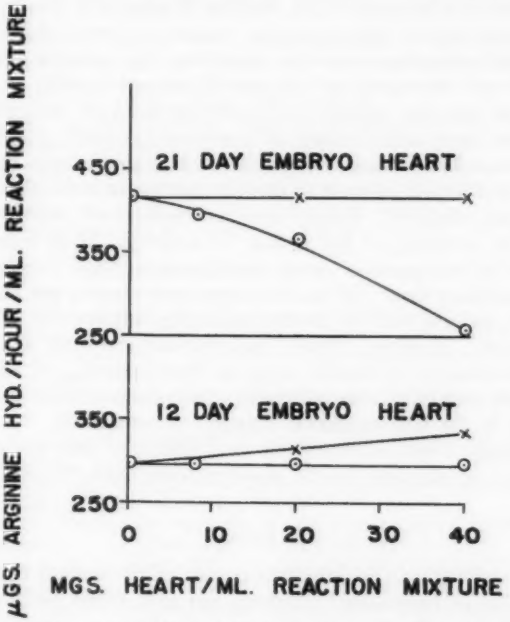

FIGURE 6. Effect of heart from 12- and 21-day embryos on the arginase activity of 4-day embryos (12.5 mg. per ml. reaction mixture) activated with 5×10^{-4} *M* Mn^{++}. ⊙ embryo-heart mixture, X embryo activity + heart activity as measured separately.

activity and inhibits 4-day embryo arginase (Fig. 6). These results show that a decrease in arginase activity is correlated with an increase in the ability to inhibit embryo arginase.

Evidence presented in this section suggests that the arginase activity expressed by tissue homogenates is determined by the concentrations of both enzyme and inhibitor(s), different tissues contain different relative amounts of inhibitor to enzyme and that in one case the developmental decrease of arginase activity is correlated with an increase in inhibitory activity. We believe these results demonstrate that inhibitor(s) plays some role in the developmental change in arginase activity.

Discussion

Results reported here suggest that ribonucleic acids could be inhibiting arginase activity in crude chick embryo homogenates. Sorm and Hrubesova (1955) found that ribonucleic acid inhibits chymotrypsin and trypsin; Moss (1952) found that ribonucleic acids inhibit arginase; Klingenberg (1952) reported that cathepsin is inhibited by ribonucleic acid; Chepinoga and Pavlovskii (1956) found that ribonucleic acid and deoxyribonucleic acid inhibit aldolase and enolase; and Zittle (1946) reported that succinic dehydrogenase is inhibited by both deoxyribonucleic acid and ribonucleic acid. A large number of cases have been reported where proteins inhibit enzyme activities (*e.g.* Swartz, Kaplan and Frech, 1956). These numerous instances where high molecular weight inhibitors have been found in various tissues and organisms raise the possibility that enzyme activities of crude homogenates are not necessarily a measure of enzyme content.

Changes in the enzymic activity of developing embryos, as measured in crude homogenates, have been demonstrated in numerous instances and it seems reasonable to believe that these changes constitute one major aspect of differentiation. It would be of considerable interest to identify the mechanisms that are responsible for these observed changes. Before we can understand such mechanisms, we must establish the meaning of the change in activity. It is conceivable that an enzyme activity, as measured in crude homogenates, could change during development in at least three ways: (1) in the amount of enzyme per unit tissue, (2) in the nature of the enzyme, and (3) in the modifying influence of substances present in the homogenate. Markert (1958) has obtained evidence that embryos produce different esterases at different stages of development. Results presented in this report suggest that in the case of arginase, substances which modify its activity may play a role in the developmental change. Consequently, it is clear that the nature of the change which is expressed as a change of enzymic activity must be established before an attempt can be made to determine the mechanism causing the change.

Summary

1. Arginase activity was measured in tissues of developing eggs from the first to the seventh day of incubation. Activity per unit moist weight or dry weight decreased in embryo, pellucida, vasculosa and vitellina.

2. Evidence was obtained that all chick tissues studied contain a high molecular weight arginase inhibitor(s) which appears to be ribonucleic acid in nature.

3. Inhibitor(s) seems to play a major role in establishing the level of arginase activity expressed by crude homogenates.

4. The decreased arginase activity of hearts from developing embryos has been correlated with an increase in inhibitor activity.

5. These results suggest that the developmental decrease in arginase activity could be the result of an increase in inhibitor concentration as well as of a decrease in enzyme concentration.

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INDEX

ATP, effect of on dinitrophenol inhibition of phosphate uptake by sea urchin embryos, 133.
ATP, electron spin resonance of, 397.
ATP-ase granules of sea urchin eggs, 63.
ATP-ase of Mytilus sperm, 327.
Abdominal ganglia of Blaberus, neurosecretory cells in, 267.
Aboul-Ela, I. A. On the food of nudibranchs, 439.
Abstracts of papers presented at the Marine Biological Laboratory, 382.
Accessory sex organs of castrate rats treated with x-rays, 68.
Acclimation, temperature, effect of on metabolism of Uca, 163, 582.
Accumulation of phosphate by sea urchin embryos, 133.
Acids, amino, in aquatic invertebrates, 371.
Acoustic orientation in cave swiftlet, 497.
Actin transformation and nucleotide interactions, 401.
Activity of arginase in chick embryos, 611.
Acto- and paramyosin from mollusc muscle, 396.
Adenocarcinoma, frog, supravital dye studies on, 419.
Adenosinetriphosphatase of Mytilus sperm, 327.
Adenosinetriphosphatase, effect of on dinitrophenol inhibition of phosphate uptake by sea urchin embryos, 133.
Adenosinetriphosphatase-bearing granules of sea urchin egg, 63.
Adsorption in olfaction, 222.
Alanine in invertebrate tissues, 371.
Alcyonaria as food for nudibranchs, 439.
Alga, new species of, 54.
Algae as food for nudibranchs, 439.
Allen, K. *See* J. W. Simpson, 371.
Allen, R. D., and L. Bunim. Changes in cytoplasmic structure following fertilization in Arbacia eggs, 403.
Allen, R. D. *See* J. L. Griffin, 394, 413.
Allocentrotus, development of, 150, 492.
Allogromia, protoplasmic movement in, 100.
Alloxanized toadfish, 420.
Alpha-particles of Spisula egg, 518.
Amebae, cell membranes of, 394.
Amino acids in aquatic invertebrates, 371.
Amphibian eggs, osmotic studies of, 458, 468.
Amphibian kidney, nuclear size in, 208.
Amphineura, reproductive cycles of, 81.
Amphipods, respiration of, 594.
Amputated mouse digits, healing of, 546.
Anaerobic survival of sea weed-inhabiting animals, 594.
Anatomy of Asymphylodora, 562.
Anatomy of Emerita larvae, 356.
Anatomy of planarian eye, 511.
Anatomy of starfish cardiac stomach, 185.
Anderson, J. M. Studies on the cardiac stomach of a starfish, Patiria, 185.
Annelid eggs, cold-treatment of, 284.
Annual Report of the Marine Biological Laboratory, 1.
Annual reproductive cycles of chitons, 81.
Antibiotics, use of in raising clam larvae, 258.
Antibodies in Tubularia, 319.
Antigenic differences between stem and hydranth in Tubularia, 319.
Arginase inhibitor in chick embryo, 611.
Arginine in invertebrate tissues, 371.
Arion as food for Orthodemus, 119.
Armstrong, P. B. Contraction of muscle induced by direct light stimulation, 392.
Arsenate, effect of on phosphate uptake of sea urchin eggs, 133.
Artemia, axenic cultivation of, 347.
Arthropods, free amino acids in, 371.
Ascorbate, role of in oxidation of CO by sea urchin eggs, 454.
Aseptic cultivation of Artemia eggs, 347.
Ashton, F. T. Germinal vesicle breakdown in the eggs of Spisula and Hydroides, 389.
Aspartic acid in invertebrate tissues, 371.
Asymphylodora, life-history of, 562.
Awapara, J. *See* J. W. Simpson, 371.
Axenic culture of Artemia, 347.

Bachmann, R. W. *See* E. P. Odum, 421.
Bacteria, destruction of bivalve larvae by, 258.
Baltus, E. *See* W. S. Vincent, 388.
Bang, F. B., and A. B. Chaet. The effect of starfish toxin on amebocytes, 403.
Barnwell, F. H. *See* F. A. Brown, Jr., 405.
Bennett, M. F., and F. A. Brown, Jr. Experimental modification of the lunar rhythm and running activity of the fiddler crab Uca, 404.

BENNETT, M. F., AND F. A. BROWN, JR. Phasing of the rhythm of running activity of the fiddler crab, 404.
BENNETT, M. F. *See* F. A. BROWN, JR., 405, 406.
BERGMANN, F., J. P. REUBEN AND H. GRUNDFEST. Actions of biogenic amines and derivatives on lobster neuromuscular transmission, 405.
BERGMANN, F. *See* J. P. REUBEN, 424.
BERNSTEIN, M. H. Functional modifications of sperm structure, 433.
Berthellina, food of, 439.
Beta-particles of Spisula egg, 518.
Bird, acoustic orientation in, 497.
Birds, photoperiodic responses of, 601.
BISCHOFF, H. W. Some observations on Chlamydomonas, 54.
Bivalve larvae, destruction of by bacteria, 258.
Blaberus, neurosecretion in, 267, 275.
BLACK, R. E., AND A. TYLER. Cytochrome oxidase and oxidation of CO in eggs of the sea urchin Strongylocentrotus, 454.
BLACK, R. E., AND A. TYLER. Effects of fertilization and development on the oxidation of CO by eggs of Strongylocentrotus and Urechis as determined by use of C-13, 443.
BLUMSTEIN, J. R. *See* C. L. CLAFF, 408, 409; F. N. SUDAK, 428, 429.
Bone healing in mouse, 546.
BOOLOOTIAN, R. A. *See* A. C. GIESE, 81.
BORGESE, T. A. *See* J. W. GREEN, 394.
Botrylloides, vascular budding in, 340.
Brachiopod, breeding season of, 202.
Branching corals, skeleton formation in, 239.
BRANHAM, J. M., AND C. B. METZ. Inhibition of fertilization and agglutination in Arbacia by an extract of Fucus, 392.
Breeding seasons of chitons, 81.
Breeding season of Lingula, 202.
BRETT, W. J., H. M. WEBB AND F. A. BROWN, JR. Contribution of locomotion to the oxygen-consumption rhythm in Uca, 405.
BRETT, W. J. *See* F. A. BROWN, JR., 406; H. M. WEBB, 430, 431.
Brine shrimp, axenic cultivation of, 347.
BROOKBANK, J. W. The presence of fertilizin haptens within the unfertilized sea urchin egg, 63.
BROWN, F. A., JR., M. F. BENNETT AND W. J. BRETT. Effects of imposed magnetic fields in modifying snail orientation, 406.
BROWN, F. A., JR., W. J. BRETT AND H. M. WEBB. Fluctuations in the orientation of the mud snail Ilyanassa in constant conditions, 406.
BROWN, F. A., JR., H. M. WEBB, M. F. BENNETT AND F. H. BARNWELL. A diurnal rhythm in response of the snail Ilyanassa to imposed magnetic fields, 405.
BROWN, F. A., JR. *See* M. F. BENNETT, 404; W. J. BRETT, 405; H. M. WEBB, 430, 431.
BRYAN, J. H. D., AND J. W. GOWEN. X-ray effects on mitotic activity of the accessory sex organs of castrate rats stimulated by testosterone propionate, 68.
BUCK, J. *See* J. F. CASE, 393, 407.
Budding in Botrylloides, 340.
Bufo, osmotic studies on eggs of, 458, 468.
BUNIM, L. *See* R. D. ALLEN, 403.
Busycon, myoglobin in odontophore of, 385, 504.
BYERS, T. F. *See* L. V. HEILBRUNN, 396.

CO, oxidation of by marine eggs, 443, 454.
Calcium-deficient uterus, 416.
Calcium deposition in corals, 239.
Calculation of olfactory thresholds, 222.
Calliopius, respiration of, 594.
Callus formation in mouse, 546.
Carbon monoxide, effects of on respiration of lamellibranch tissues, 125.
Carbon monoxide, oxidation of by marine eggs, 443, 454.
Cardiac stomach of Patiria, 185.
CARLSON, A. D. Neural involvement in the firefly pseudoflash, 407.
Cartilaginous odontophore of Busycon, 385, 504.
CASCARANO, J. *See* E. KIVY-ROSENBERG, 415.
CASE, J. F., AND J. BUCK. Central nervous aspects of firefly excitation, 393.
CASE, J. F., AND J. BUCK. Peripheral aspects of firefly excitation, 407.
Castrate rats, x-irradiation of, 68.
Cation exchange in mackerel erythrocytes, 394.
Cave swiftlet, acoustic orientation of, 497.
Cell division of Spisula eggs, 518.
Cercariae of Asymphylodora, 562.
CESKA, M., AND J. R. FISHER. An arginase inhibitor(s) and its possible role in the developmental decrease of arginase activity in chick embryos, 611.
Ceylon swiftlets, acoustic orientation in, 497.
CHAET, A. B., AND R. A. MCCONNAUGHY. Physiologic activity of nerve extracts, 407.
CHAET, A. B. *See* F. B. BANG, 403.
Chaetopterus eggs, exaggerated elevation of fertilization membrane of, 284.
Chemoreception in humans, 222.

CHENEY, R. H., AND C. C. SPEIDEL. Relation of x-ray dosage to modifications in fertilization and early development of Arbacia, 408.
CHENEY, R. H. *See* C. C. SPEIDEL, 427.
Chick embryos, arginase activity in, 611.
Chiloscyllium, histochemistry of egg capsules of, 298.
Chitons, reproductive cycles of, 81.
Chlamydomonas, new species of, 54.
Chlormerodrin, distribution of in fish tissue, 251.
Chromatography of invertebrate tissues, 371.
Chromatography of shark egg capsules, 298.
Chromatophorotropins in Cambarellus, 382.
Chromodorids, food of, 439.
Chromosome number of new species of Chlamydomonas, 54.
CHUANG, S. H. The breeding season of the brachiopod, Lingula, 202.
CLAFF, C. L., F. N. SUDAK, J. R. BLUMSTEIN AND F. J. TEYAN. A rapid method for determining respiratory quotients in small animals, 408.
CLAFF, C. L., F. N. SUDAK, F. J. TEYAN AND J. R. BLUMSTEIN. Metabolism-body temperature relationships in cold acclimatized rats treated with 2,4-D, 409.
CLAFF, C. L. *See* F. N. SUDAK, 428, 429; F. J. TEYAN, 429.
Clam egg, vital staining of, 518.
Clam larvae, destruction of by marine bacteria, 258.
Clam larvae grown at different temperatures, 308.
Cleavage of Allocentrotus eggs, 150, 492.
Cleavage in Arbacia, effects of purine analogs on, 436.
Cleavage of cold-treated Chaetopterus eggs, 284.
Cleavage of sea urchin eggs, penetration of ATP during, 133.
Cleavage of Spisula egg, 518.
Cleavage-blocking action of sea urchin antisera, 63.
Cocoons of Cynthia, 482.
Coelenterate, antigens in, 319.
Coelenterates, free amino acids in, 371.
Cold, effect of on metabolism of Uca, 582.
Cold, effect of on uptake of phosphate by sea urchin embryos, 133.
Cold-treatment of Chaetopterus eggs, 284.
Collocalia, echolocation in, 497.
Color changes in shark egg capsules, 298.
CONNELL, M. G. Nuclear sizes in Rana mesonephroi, 208.
Contraction of muscle induced by light stimulation, 392.
CONWAY, D. M., AND T. SAKAI. The irreversible effects of caffeine on striated muscles, 409.
CONWAY, D. M., AND T. SAKAI. Latency period, contraction time and fiber diameter, 410.
CONWAY, D. M., AND T. SAKAI. The sensitivity of the non-propagating muscle to longitudinal current, 410.
COOPERSTEIN, S. J. *See* A. LAZAROW, 418.
Corals, skeleton formation in, 239.
Corpora cardiaca of roach, neurosecretion from, 275.
Cortical granule-hyaline material of Arbacia, 394.
COSTELLO, D. P. A general theory of membrane elevation in marine eggs, 410.
COSTELLO, D. P., AND C. HENLEY. A fundamental difference in the vitelline membranes of the eggs of marine annelids, 411.
COSTELLO, D. P. *See* C. HENLEY, 414.
COUSINEAU, G. *See* P. R. GROSS, 413.
Crab, sand, larval development of, 356.
Crabs, fiddler, metabolism of, 163, 582.
Crassostrea, cytochrome system in, 125.
Crustacean, axenic culture of, 347.
Crustacean, metabolism of, 163, 582.
Crustacean, taxonomy of, 154.
CSAPO, A. I. *See* H. A. KURIYAMA, 416, 417; J. M. MARSHALL, 419.
Culture of Artemia, 347.
Culture of Chlamydomonas, 54.
Culture of Emerita, 356.
Cushion star, cardiac stomach of, 185.
Cyanide, effect of on respiration of lamellibranch tissues, 125.
Cycles, reproductive, of chitons, 81.
Cynthia silkworm, respiratory metabolism of, 482.
Cytochrome oxidase, role of in oxidation of CO by marine eggs, 454.
Cytochrome oxidase system of Busycon odontophore, 385, 504.
Cytochrome system in Cynthia silkworm, 482.
Cytochrome system in marine lamellibranchs, 125.
Cytology of Chlamydomonas, 54.
Cytology of cold-treated Chaetopterus eggs, 284.
Cytology of x-irradiated castrate rats, 68.
Cytoplasm of Arbacia eggs, effect of sulfhydryl compounds on, 425.
Cytoplasmic division of Allocentrotus egg, 150.
Cytoplasmic inclusions in amebae, 382.
Cytoplasmic movement in Allogromia, 100.

DNA in frog mesonephroi, 208.
DNA in x-irradiated castrate rats, 68.
Dark, effect of on oxidation of CO by marine eggs, 443, 454.
Darkness, role of in photoperiodic responses of birds, 601.
DAVIES, J. T., AND F. H. TAYLOR. The role of adsorption and molecular morphology in olfaction: the calculation of olfactory thresholds, 222.
Decrease of arginase activity in chick embryos, 611.
Dehydrogenases in Echinarachnius eggs and embryos, 420.
Denuding of Chaetopterus eggs, 284.
Deposition of calcium in corals, 239.
Dermal healing in mouse, 546.
Dermatobranchus, food of, 439.
Desoxyribosenucleic acid in frog mesonephroi, 208.
Desoxyribonucleic acid in x-irradiated castrate rats, 68.
Destruction of bivalve larvae by bacteria, 258.
Developing marine eggs, oxidation of CO by, 443, 454.
Development of Allocentrotus, 150, 492.
Development of Botrylloides colonies, 340.
Development of clam larvae in presence of bacteria, 258.
Development of cold-treated Chaetopterus eggs, 284.
Development of Emerita, 356.
Development of podophyllin-treated Chaetopterus eggs, 415.
Development of Venus larvae, 308.
Developmental decrease of arginase activity in chick embryos, 611.
Digenetic trematode, life-history of, 562.
Digestion in Orthodemus, 119.
Digestion in Patiria, 185.
Digits of mice, wound healing in, 546.
Dinitrophenol, effect of on phosphate uptake of sea urchin embryos, 133.
Discodoris, food of, 439.
Distribution of diuretic in fish tissues, 251.
Diuretic, distribution of in fish tissues, 251.
Diurnal rhythm in Ilyanassa, in response to magnetic fields, 405.
Dogfish, distribution of diuretic in tissues of, 251.
Dugesia, eye of, 511.

EARTHWORM as food for Orthodemus, 119.
Echinoderm, development of, 150, 492.
Echinoderm cardiac stomach, 185.
Echinoderm egg, fertilizin haptens in, 63.
Echinoderm eggs, oxidation of CO by, 443, 454.
Echinoderm embryos, phosphate accumulation by, 133.
Echinoderms, free amino acids in, 371.
Echolocation in Collocalia, 497.
Ecology of chitons, 81.
Ecology of corals, 239.
Ecology of isopods, 154.
Ecology in relation to respiration of some invertebrates, 601.
EDMONDS, M., AND J. S. ROTH. Purification and properties of a ribonuclease from squid, 393.
Effects of temperature on development of Allocentrotus, 150.
Effects of x-rays on castrate rats, 68.
Egg, sea urchin, fertilizin haptens in, 63.
Egg of Allocentrotus, development of, 150, 492.
Egg capsules of shark, histochemistry of, 298.
Egg-jelly precipitating agents from Arbacia sperm, 416.
Egg membranes, 410, 411.
Eggs, amphibian, osmotic studies of, 458, 468.
Eggs, Chaetopterus, exaggerated elevation of fertilization membrane of, 284.
Eggs, marine, oxidation of CO by, 443, 454.
EISEN, J. D. The physiology of the predator-prey relationship existing between Paramecium and Didinium, 383.
Electrical activity of Limulus nerve and ganglion, 397.
Electrical stimulation of roach, 275.
Electron microscope study of planarian eye, 511.
Electron microscopes, correction of astigmatism in, 423.
Electron microscopy of amphibian tumor, 386.
Electron microscopy of Arbacia sperm, 433.
Electron microscopy of Crassostrea sperm, 386.
Electron miscroscopy of Limulus gill cartilage, 398.
Electron microscopy of Limulus muscle, 387.
Electron microscopy of Limulus sperm, 423.
Electron microscopy of regenerating rat liver, 413.
Electron microscopy of Schistosoma, 387.
Elevation of Chaetopterus fertilization membrane, 284.
Embryology of Allocentrotus, 150, 492.
Embryology of cold-treated Chaetopterus eggs, 284.
Embryos, chick, arginase activity in, 611.
Embryos, marine, oxidation of CO by, 443, 454.

Embryos, sea urchin, phosphate accumulation by, 133.
Emerita, larval development of, 356.
Enoplus, respiration of, 594.
Enzymatic control of phosphate uptake in sea urchin embryos, 133.
Enzyme activity of marine eggs, 454.
Enzyme inhibition in chick embryos, 611.
Enzyme kinetics of Mytilus sperm extracts, 327.
Epidermal healing in mouse, 546.
Estrogen and progesterone, effects of on rat uterine muscle fibers, 391.
Exaggerated elevation of Chaetopterus fertilization membrane, 284.
Excitability responses of lobster muscle fibers, 431, 432.
Excitation in firefly, 393, 407.
Excitation in nerve and muscle, 391.
Excitation of presynaptic terminals at lobster neuromuscular junctions, 424.
Eye, planarian, electron microscope study of, 511.

FAT deposition in birds, in relation to light, 601.
FAUST, R. G., R. F. JONES AND A. K. PARPART. Isolation and characterization of cortical granule-hyaline material of the Arbacia egg, 394.
FEIGELSON, M. *See* P. R. GROSS, 413.
Fertilization, changes in cytoplasm of Arbacia eggs after, 403.
Fertilization, effects of on oxidation of CO by marine eggs, 443.
Fertilization, effect of on uptake of phosphate by sea urchin embryos, 133.
Fertilization, inhibition of by Fucus extract, 392.
Fertilization inhibition by Arbacia dermal secretion, 398.
Fertilization membrane of Chaetopterus egg, exaggerated elevation of, 284.
Fertilization of Spisula egg, 518.
Fertilized amphibian eggs, osmotic properties of, 458, 468.
Fertilizin, effects of sperm on, 395.
Fertilizin haptens of unfertilized sea urchin egg, 63.
Fiddler crabs, metabolism of, 163, 582.
Filament protoplasmic streaming in Allogromia, 100.
FINE, A. S. *See* P. PERSON, 385, 504.
FINGERMAN, M. Physico-chemical characterization of chromatophorotropins in the crayfish Cambarellus, 382.
FINGERMAN, M., W. C. MOBBERLEY, JR. AND C. W. PHILPOTT. Electrophoretic analysis of the distal retinal pigment dark-adapting and light-adapting hormones in the prawn Palaemonetes, 411.
FINGERMAN, M., C. W. PHILPOTT, M. I. SANDEEN AND W. C. MOBBERLEY, JR. Influence of long exposure to light and darkness upon the distal retinal pigment light-adapting hormone of the prawn Palaemonetes, 412.
FINGERMAN, M., M. I. SANDEEN, W. C. MOBBERLY, JR. AND C. W. PHILPOTT. Hormonal regulation of the distal retinal pigment of the shrimp Crangon, 412.
FINGERMAN, M. *See* M. I. SANDEEN, 425.
FIORENTINO, E. *See* J. S. ROTH, 398.
Firefly, excitation in, 393, 407.
Fish, marine, distribution of diuretic in tissues of, 251.
Fish, thyroid function in, 89.
FISHER, F. M., JR. *See* R. C. SANBORN, 399.
FISHER, J. R. *See* M. CESKA, 611.
Flagellar movements of Mytilus sperm, 327.
Flatworm, nutrition of, 119.
Flounder, distribution of diuretic in tissues of, 251.
Fluctuations in rate of locomotion of Ilyanassa, 431.
Fluorescence, induced, in marine eggs, 429.
Follicular layers of amphibian eggs, role of in osmotic properties, 468.
Food of nudibranchs, 439.
Foraminiferan, protoplasmic movement in, 100.
Formation of shark egg capsules, 298.
Formation of skeleton in corals, 239.
Free amino acids in aquatic invertebrates, 371.
Frog, healing process in, 546.
Frog kidney, nuclear size in, 208.
Frogs, osmotic studies on eggs of, 458, 468.
Function of thyroid in Fundulus, 89.
Fundulus, thyroid function in, 89.

GALTSOFF, P. S., AND D. E. PHILPOTT. Electron microscope study of the sperm of Crassostrea, 386.
Gametes of chitons, 81.
Gametes of Lingula, 202.
Gammarus, respiration of, 594.
Gangliar neurosecretory cells in Blaberus, 267.
GELDIAY, S. Neurosecretory cells in ganglia of the roach Blaberus, 267.
GELDIAY, S. *See* E. S. HODGSON, 275.
Geographical factors in relation to metabolism of Uca, 163, 582.
Gephyrean eggs, oxidation of CO by, 443.
Germinal vesicle breakdown in Spisula and Hydroides eggs, 389.

GIESE, A. C., J. S. TUCKER AND R. A. BOOLOOTIAN. Annual reproductive cycles of the chitons Katherina and Mopalia, 81.
GILL, E. W. *See* A. S. KUPERMAN, 390.
Glutamic acid in invertebrate tissues, 371.
Glycerinated protoplasm, 388.
Glycine in invertebrate tissues, 371.
Gnorimosphaeroma, taxonomy of, 154.
Gonad indices of chitons, 81.
Gonad weights of birds, in relation to light, 601.
GONSE, P. H. Respiration and motility in Spisula spermatozoa, 433.
GOREAU, T. F., AND N. I. GOREAU. The physiology of skeleton formation in corals. II., 239.
GOWEN, J. W. *See* J. H. D. BRYAN, 68.
GREEN, J. W., G. HOLSTEN AND T. A. BORGESE. Cation exchange in the erythrocytes of the mackerel, Scomber, 394.
GREIF, R. L., AND M. DU VIGNEAUD. Distribution of a radiomercury-labelled diuretic (chlormerodrin) in tissues of marine fish, 251.
GRIFFIN, J. L. Isolation and chemical identification of the crystalline cytoplasmic inclusions in the large, free-living amoebae, 382.
GRIFFIN, J. L., AND R. D. ALLEN. Movements of the cell membrane of some fresh- and salt-water amebae, 394.
GRIFFIN, J. L., AND R. D. ALLEN. A reliable collecting locality for Pelomyxa, 413.
GROSS, P. R., AND G. COUSINEAU. Glucose-6-phosphatase in sea urchin eggs, 413.
GROSS, P. R., M. FEIGELSON AND D. E. PHILPOTT. Ultrastructural changes in regenerating rat liver, 413.
GROSS, P. R., W. SPINDEL AND D. E. PHILPOTT. The block to cell division produced by deuterium oxide, 395.
GROSS, S. R. Functional complementarity of leucine mutants of Neurospora, 414.
Growth of clam larvae in presence of bacteria, 258.
Growth of Venus larvae, 308.
Growth rate of corals, 239.
GRUNDFEST, H. *See* F. BERGMANN, 405; J. P. REUBEN, 424; R. WERMAN, 431, 432.
GUILLARD, R. R. L. Further evidence of the destruction of bivalve larvae by bacteria, 258.
GUTTMAN, R., AND L. ZABLOW. Membrane resistance and rectification in muscle stimulated by rapid cooling, 414.

HALACARUS, respiration of, 594.
Hapten sites in Tubularia, 319.
Haptens of unfertilized sea urchin egg, 63.
Hard shell clam larvae grown at different temperatures, 308.
HARRIS, P. J. A study of thyroid function in Fundulus, 89.
HASKELL, J. A. *See* R. C. SANBORN, 399.
HATHAWAY, R. R. The effects of sperm on S^{35}-labelled Arbacia fertilizin, 395.
HAYASHI, T., R. ROSENBLUTH AND H. C. LAMONT. Studies of fibers of acto- and paramyosin from lamellibranch muscle, 396.
HAYASHI, T. *See* K. K. TSUBOI, 401.
Healing processes in mice, 546.
Heart, molluscan, cytochromes in, 125.
Heart of chick embryos, arginase activity of, 611.
Heat, effect of on metabolism of Uca, 163, 582.
HEILBRUNN, L. V. The action of glycerol on protoplasm, 388.
HEILBRUNN, L. V., AND T. F. BYERS. A study of membrane elevation in various marine eggs, 396.
Hemolytic accelerating power of various substances, 222.
HENLEY, C. Exaggerated elevation of the fertilization membrane of Chaetopterus eggs, resulting from cold-treatment, 284.
HENLEY, C. Shape changes in fertilized Chaetopterus eggs treated with podophyllin, 415.
HENLEY, C., AND D. P. COSTELLO. The effects of podophyllin on the fertilized eggs of Chaetopterus, 414.
HENLEY, C. *See* D. P. COSTELLO, 411.
Hermatypic corals, skeleton formation in, 239.
Hexabranchus, food of, 439.
High temperature, effect of on metabolism of Uca, 163, 582.
Histochemistry of Ephelota, 435.
Histochemistry of shark egg capsules, 298.
Histochemistry of Spisula egg, 518.
Histochemistry of starfish cardiac stomach, 185.
Histology of Fundulus thyroid, 89.
Histology of healing process in mouse digits, 546.
Histology of neurosecretory cells in Blaberus, 267, 275.
Histology of Orthodemus, 119.
Histology of planarian eye, 511.
Histology of starfish cardiac stomach, 185.
HODGSON, E. S., AND S. GELDIAY. Experimentally induced release of neurosecretory materials from roach corpora cardiaca, 275.
HOLSTEN, G. *See* J. W. GREEN, 394.

HOLZ, G. G., JR. *See* C. C. SPEIDEL, 384.
Homothallic sexual reproduction of Chlamydomonas, 54.
Hormones of Crangon, 412, 425.
Hormones, of Palaemonetes, 411, 412.
Hormones, effect of on x-irradiated castrate rats, 68.
HUNTER, F. R., AND O. DE LUQUE. Osmotic studies of amphibian eggs. II., 468.
HUNTER, F. R. *See* O. DE LUQUE, 458.
Hyale, respiration of, 594.
Hybrids of sea urchins, 492.
Hydranth antigens in Tubularia, 319.
Hydrocorallines, skeleton formation in, 239.
Hydroid, antigens in, 319.
Hyla, osmotic studies on eggs of, 458, 468.
Hyperactivity of roach, relation of to neurosecretion, 275.
Hyperglycemic agents, responses of Raja to, 433.
Hyperpolarization of lobster muscle fibers, 424.
Hyperthyroid Fundulus, 89.
Hypothermia in rat, 428.

IMMUNOLOGICAL studies on sea urchin egg, 63.
Immunological studies on Tubularia, 319.
Indole acetic acid treatment of fresh-water plants, 418.
Inhibitor, arginase, in chick embryos, 611.
Inhibitory action of phenylthiourea on respiratory metabolism of Cynthia, 482.
Inorganic pyrophosphate, effect of on Mytilus sperm, 327.
Invertebrates, sea weed-inhabiting, respiration of, 594.
Irradiation of castrate rats, 68.
ISENBERG, I., AND A. SZENT-GYÖRGYI. On the electron spin resonance of adenosine triphosphate, 397.
Isopod, taxonomy of, 154.

JAHN, T. L., AND R. A. RINALDI. Protoplasmic movement in the foraminiferan, Allogromia: and a theory of its mechanism, 100.
Janus Green B Staining of Spisula egg, 518.
Jelly layer, role of in osmotic properties of amphibian eggs, 468.
JENNINGS, J. B. Observations on the nutrition of the land planarian Orthodemus, 119.
JOHNSON, W. H., AND A. G. SZENT-GYÖRGYI. The molecular basis for the "catch" mechanism in molluscan muscles, 382.
JONES, B. M., AND R. S. WILSON. Studies on the action of phenylthiourea on the respiratory metabolism and spinning behaviour of the Cynthia silkworm, 482.
JONES, R. F. *See* R. G. FAUST, 394.
Junco, photoperiodic responses of, 601.

KANWISHER, J. *See* W. WIESER, 594.
Katherina, reproductive cycles of, 81.
KAWAI, K. The cytochrome system in marine lamellibranch tissues, 125.
Kidney of frog, nuclear size in, 208.
Kidneys of marine fish, 251.
Kinetics, enzyme, of Mytilus sperm extracts, 327.
Kinetics of enzyme activity in chick embryos, 611.
KIVY-ROSENBERG, E., J. CASCARANO AND G. MERSON. Examination of some DPN- and TPN-dependent dehydrogenase activity before and after relatively high doses of x-irradiation of Spisula eggs, 415.
KOHLER, K., AND C. B. METZ. Extraction of egg jelly precipitating factors from Arbacia sperm, 416.
KRISHNAN, K. Histochemical studies on the nature and formation of egg capsules of the shark Chiloscyllium, 298.
KUPERMAN, A. S., G. WERNER AND E. W. GILL. Presynaptic activity on drug-induced neuromuscular facilitation, 390.
KURIYAMA, H. A., AND A. I. CSAPO. The calcium-deficient uterus, 416.
KURIYAMA, H. A., AND A. I. CSAPO. The "evolution" of membrane and myoplasmic activity of uterine muscle, 417.

LABORATORY culture of Artemia, 347.
Laboratory culture of Emerita, 356.
Lamellibranchs, cytochrome system in, 125.
LAMONT, H. C. *See* T. HAYASHI, 396.
Land planarian, nutrition of, 119.
Larvae of Allocentrotus, 150.
Larvae of Artemia, 347.
Larvae of Blaberus, neurosecretion in, 267.
Larvae of Cynthia, respiratory metabolism of, 482.
Larvae of Venus, destruction of by bacteria, 258.
Larvae of Venus, size and shape of, 308.
Larval development of Emerita, 356.
LASH, J. W. *See* P. PERSON, 385, 504.
Latitudinal adaptation of Uca, 163, 582.
LAZAROW, A., P. MAKINEN AND S. J. COOPERSTEIN. Glucose-6-phosphatase content of toadfish islet tissue, 418.
LEONE, V. The influence of ribonuclease on the development of the sea urchin, 434.

LEONE, V. Some structures found in electron microscope studies of an amphibian tumour, 386.
Leucothrix, culture of, 418.
LEWIN, R. A. Leucothrix mucor, 418.
LEWIN, R. A. Uptake of strontium by Syracosphaera, 384.
Life-history of Asymphylodora, 562.
Ligation of Blaberus nerve cord, 267.
Light, effect of on oxidation of CO by marine eggs, 443, 454.
Light, effect of on skeleton formation in corals, 239.
Light, role of in photoperiodic responses of birds, 601.
Light receptors of planarian eye, 511.
Light in relation to orientation of cave swiftlets, 497.
Lingula, breeding season of, 202.
Lipids of Arbacia eggs and embryos, 435.
LITCHFIELD, J. B., AND A. H. WHITELEY. Studies on the mechanism of phosphate accumulation by sea urchin embryos, 133.
Locomotion of Ilyanassa, effects of imposed magnetic fields on, 406, 430.
LOOSANOFF, V. L. The size and shape of metamorphosing larvae of Venus grown at different temperatures, 308.
DE LORENZO, A. J. Fine structure of synapses, 390.
Low temperature, effect of on metabolism of Uca, 582.
Low temperature, effect of on phosphate uptake of sea urchin embryos, 133.
Low temperature, effects of on Chaetopterus eggs, 284.
LOWENHAUPT, B. The chemistry of ion transport and its relation to excitation in nerve and muscle, 391.
LOWENHAUPT, B. The effect of indole acetic acid and serotonin on the calcium-45 content of leaves of the fresh-water plants, Potamogeton and Elodea, 418.
DE LUQUE, O., AND F. R. HUNTER. Osmotic studies of amphibian eggs. I., 458.
DE LUQUE, O. *See* F. R. HUNTER, 468.
Lysosomes, 385.
Lytechinus eggs, fertilizin haptens in, 63.

MACTRA (Spisula) egg, vital staining of, 518.
Magnesium ion, effects of on Mytilus sperm extracts, 327.
Magnetic fields, effects of in modifying snail orientation, 406, 430.
MAKINEN, P. *See* A. LAZAROW, 418.
Marine bacteria, destruction of clam larvae by, 258.
Marine fish, distribution of diuretic in, 251.
Marine lamellibranchs, cytochrome system in, 125.
Marine snail, myoglobin in odontophore of, 385, 504.
MARSH, J. B. A rapid method for measuring total lipid, applied to the analysis of the lipids and lipoproteins of Arbacia eggs and embryos, 435.
MARSHALL, J. M. Effects of estrogen and progesterone on single uterine muscle fibers in the rat, 391.
MARSHALL, J. M., AND A. I. CSAPO. The effects of calcium deficiency and oxytocin on the membrane activity of uterine muscle, as measured by the sucrose-gap method, 419.
Massive corals, skeleton formation in, 239.
MATEYKO, G. M. Supravital dye studies on isolated cells of frog renal adenocarcinoma, 419.
Maturation of Spisula egg, 518.
MAVRIDIS, P. J., AND R. C. SANBORN. Effect of temperature on electrical activity of nerve cord and cardiac ganglion of Limulus, 397.
MCCONNAUGHY, R. A. *See* A. B. CHAET, 407.
Mechanism of phosphate accumulation by sea urchin embryos, 133.
Megalops of Emerita, 356.
Melanophore-dispersing action of ataraxic drugs, 400.
Membrane, fertilization, of Chaetopterus egg, 284.
Membrane elevation in marine eggs, 396, 410, 411.
Mercenaria, growth of larvae of, 308.
MERSON, G. The localization of some dehydrogenases in Echinarachnius, 420.
MERSON, G. *See* E. KIVY-ROSENBERG, 415.
Mesonephroi of Rana, nuclear size in, 208.
Metabolism-body temperature relations in cold acclimatized rats treated with 2,4-D, 409.
Metabolism of Busycon odontophore, 504.
Metabolism of Cynthia silkworm, 482.
Metabolism of marine eggs, 443, 454.
Metabolism of marine lamellibranchs, 125.
Metabolism of sea urchin eggs, 133.
Metabolism of sea weed-inhabiting animals, 594.
Metabolism of Uca, 163, 582.
Metacercariae of Asymphylodora, 562.
Metamorphosing larvae of Venus, 308.
Metanauplii of Artemia, 347.
Methylene blue staining of Spisula egg, 518.
METZ, C. B. Studies on the fertilization inhibiting action of Arbacia dermal secretion, 398.

METZ, C. B. *See* J. M. BRANHAM, 392; K. KOHLER, 416.
Microhalophilic species of Chlamydomonas, 54.
Migratory birds, photoperiodic responses of, 601.
Mites, sea weed-inhabiting, respiration of, 594.
Mitosis of Spisula egg, 518.
Mitotic activity of x-irradiated castrate rats, 68.
Mitotic apparatus of Chaetopterus egg, effect of urethan on, 399.
Mitotic block induced by deuterium oxide, 395.
Mitotic inhibition in sea urchin egg, 437.
MOBBERLEY, W. C., JR. *See* M. FINGERMAN, 411, 412.
Molecular morphology in olfaction, 222.
Mollusc, myoglobin in odontophore of, 385, 504.
Mollusc, reproductive cycles of, 81.
Mollusc egg, vital staining of 518.
Mollusc larvae, destruction of by bacteria, 258.
Mollusc larvae grown at different temperatures, 308.
Mollusc sperm, ATP-ase of, 327.
Molluscs, cytochrome system in, 125.
Molluscs, free amino acids in, 371.
MOORE, A. R. Some effects of temperature on development in the sea urchin Allocentrotus, 150.
MOORE, A. R. On the embryonic development of the sea urchin Allocentrotus, 492.
Mopalia, reproductive cycles of, 81.
Morphogenesis of chick, arginase activity during, 611.
Morphology of Asymphylodora, 562.
Morphology of Chlamydomonas, 54.
Morphology of isopod, 154.
Morphology of molecules, in relation to olfaction, 222.
Morphology of Patiria cardiac stomach, 185.
Morphology of shark egg capsules, 298.
Morphology of Venus larvae, 308.
MORRILL, J. B., JR. Antigenic differences between stem and hydranth in Tubularia, 319.
MOULE, M. *See* P. F. NACE, 420.
Mouse, healing processes in, 546.
Movement, protoplasmic, in Allogromia, 100.
Muscle, cooled, membrane resistance and rectification in, 414.
Muscle, non-propagating, sensitivity of to longitudinal current, 410.
Muscle, striated, effect of caffeine on, 409.
Muscle, uterine, effects of calcium deficiency and oxytocin on, 419.
Muscle, uterine, latency period in, 410.
Muscle, uterine, membrane and myoplasmic activity of, 417.
Muscle contraction in molluscs, 382.
Muscular activity in rats treated with 2,4-D, 428.
Musculature of starfish cardiac stomach, 185.
Mustelus, distribution of diuretic in tissues of, 251.
Myoglobin, possible role of in oxygen transport, 402.
Myoglobin in Busycon odontophore, 385, 504.
Mytilus, cytochrome system in, 125.
Mytilus sperm, ATP-ase of, 327.

NACE, P. F., C. YIP, I. SILKOVSKIS, M. MOULE AND J. MOULE. Early responses of alloxanized toadfish, 420.
NASS, S. Phosphoprotein phosphatase in the ovary of Rana, 420.
NELSON, L. ATP-ase of Mytilus spermatozoa. II., 327.
NELSON, L. Inorganic pyrophosphatase and the motility of spermatozoa, 435.
Nematode, life-cycle and metabolism of, 383.
Nematodes, sea weed-inhabiting, respiration of, 594.
Neural involvement in firefly pseudoflash, 407.
Neuromuscular facilitation, 390.
Neuromuscular mechanisms of sound production in Opsanus, 438.
Neuromuscular transmission in lobster, 405.
Neurosecretion in Blaberus, 267, 275.
Neurosecretory organs of crayfish, paper chromatography of, 426.
Neurospora, leucine mutants of, 414.
Neutral red staining of Spisula egg, 518.
New species of Chlamydomonas, 54.
New species of digenetic trematode, 562.
Nidamental glands of shark, histochemistry of, 298.
NOVICK, A. Acoustic orientation in the cave swiftlet, 497.
NOVIKOFF, A. B. Lysosomes and the physiology and pathology of cells, 385.
Nuclear reactions of x-irradiated castrate rats, 68.
Nuclear sizes in Rana mesonephroi, 208.
Nudibranchs, food of, 439.
Nutrition of Artemia, 347.
Nutrition of nudibranchs, 439.
Nutrition of Orthodemus, 119.

ODONTOPHORE of Busycon, 385, 504.
Odor perception, 222.
ODUM, E. P., AND R. W. BACHMANN. Uptake of Zn-65 and primary productivity of marine benthic algae, 421.
OKA, H., AND H. WATANABE. Vascular budding in Botrylloides, 340.

Olfaction, adsorption and molecular morphology in, 222.
Opsanus, distribution of diuretic in tissues of, 251.
Orientation, acoustic, of cave swiftlets, 497.
Orientation of mud snail, 406.
Orthodemus, nutrition of, 119.
Osmoregulation in Fundulus, 89.
Osmoregulation in isopods, 154.
Osmotic studies of amphibian eggs, 458, 468.
OSTERHOUT, W. J. V. Protoplasmic contraction and cell wall swelling in a marine alga, 421.
OSTERHOUT, W. J. V. Water relations in injury to Nitella cells, 422.
Ova, marine, oxidation of CO by, 443, 454.
Ova, sea urchin, fertilizin haptens in, 63.
Oxidation of CO by marine eggs, 443, 454.
Oxygen consumption of Uca, 163, 582.
Oxygen consumption in Uca before and after eyestalk removal, 429.
Oxygen requirements of sea weed-inhabiting animals, 594.
Oxygen uptake of Busycon odontophore, 504.
Oxygen uptake of Cynthia silkworm, 482.
Oxygen uptake of marine eggs, 443, 454.
Oxygen uptake of marine lamellibranchs, 125.

PALINSCAR, E. E. Effects of some purine analogs on cleavage in Arbacia, 436.
PALINSCAR, E. E. Histochemical studies of the reproductive cycle of Ephelota, 435.
PAPACONSTANTINOU, J. A study of the soluble proteins of the adult squid lens, 422.
Paralycthys, distribution of diuretic in tissues of, 251.
PARPART, A. K. *See* R. G. FAUST, 394.
Parthenogenetic activation of sea urchin eggs, 133.
Patiria, cardiac stomach of, 185.
Pelomyxa, collecting locality for, 413.
Penicillin, use of in raising clam larvae, 258.
Permeability of amphibian eggs, 458, 468.
PERSON, P., J. W. LASH AND A. S. FINE. On the occurrence of myoglobin and cytochrome oxidase activity in the cartilagionus odontophore of the marine snail Busycon, 385, 504.
PERSON, P., D. PHILPOTT AND E. SUBEN. A light and electron microscope study of Limulus gill cartilage, 398.
Pharynx, role of in feeding of Orthodemus, 119.
Phenolase in Cynthia silkworm, 482.
Phenylthiourea, action of on Cynthia silkworm, 482.
Philippine swiftlets, acoustic orientation in, 497.
PHILPOTT, C. W. *See* M. FINGERMAN, 411, 412.
PHILPOTT, D. E. A calculator to aid in the correction of astigmatism in electron microscopes, 423.
PHILPOTT, D. E., AND W. N. SHAW. An electron microscope study of the sperm of Limulus, 423.
Phosphatase content of toadfish islet tissue, 418.
Phosphatase in ovary of Rana, 420.
Phosphatase in sea urchin eggs, 413.
Phosphate accumulation by sea urchin embryos, 133.
Photoperiodic responses of birds, 601.
Photoreceptors of planarian eye, 511.
Phyllodesmium, food of, 439.
Phylogeny of Digenea, 562.
Physiological variation in Uca, 163, 582.
Physiology of skeleton formation in corals, 239.
Pinctada, cytochrome system in, 125.
Planarian, land, nutrition of, 119.
Planarian eye, electron microscope study of, 511.
Plasmagel of Allogromia, 100.
Plasmalogen structure, 387.
Platyhelminth, eye of, 511.
Pleuroleura, food of, 439.
Plutei of Allocentrotus, 150, 492.
Podophyllin, effects of on Chaetopterus eggs, 414, 415.
Polyploidy in frog mesonophroi, 208.
Polyploidy in Mormoniella, 402.
Potassium content of amphibian eggs, 468.
Predator-prey relationship between Paramecium and Didinium, 383.
PRESS, N. Electron microscope study of the distal portion of a planarian retinular cell, 511.
Productivity of marine benthic algae, 421.
Progenesis among digenetic trematodes, 562.
Prostate of x-irradiated castrate rats, 68.
Proteins of adult squid lens, 422.
Proteins of sea urchin embryos, 427, 436.
Protoplasm, action of glycerol on, 388.
Protoplasm, electrophoretic studies on, 389.
Protoplasmic contraction in marine alga, 421.
Protoplasmic movement in Allogromia, 100.
Protozoan, protoplasmic movement in, 100.
PROVASOLI, L., AND K. SHIRAISHI. Axenic cultivation of the brine shrimp Artemia, 347.
Pseudomonas, destruction of clam larvae by, 258.
Pyridine hemochrome of Busycon odontophore, 504.

Pyrophosphatase, effect of on motility of sperm, 435.
Pyrophosphate, effect of on Mytilus sperm, 327.

QUINONE-TANNING of shark egg capsule, 298.

RNA, incorporation of radioactive label into, 388.
RNA as possible arginase inhibitor, 611.
Radiation-induced mitotic delay, 437.
Radioactive calcium, use of in studying skeleton formation of corals, 239.
Radioactive carbon, use of in study of oxidation of CO by marine eggs, 443, 454.
Radioactive iodine, treatment of Fundulus with, 89.
Radiomercury-labelled diuretic, distribution of in fish tissues, 251.
Radiophosphorus accumulation by sea urchin embryos, 133.
Radiophosphorus uptake of Fucus, 430.
Rana mesonephroi, nuclear size in, 208.
RANZI, S. On the stability of the structures of animalized and vegetalized sea urchin embryos, 436.
RAPPORT, M. M. Present status of the problem of plasmalogen structure, 387.
Rats, x-irradiation of, 68.
Reaggregations in tunicates, 426.
REBHUN, L. I. Studies of early cleavage in the surf-clam Spisula, using methylene blue and toluidine blue as vital stains, 518.
Rediae of Asymphylodora, 562.
Reef corals, skeleton formation in, 239.
REES, G. H. Larval development of the sand crab Emerita in the laboratory, 356.
Regeneration of Fundulus thyroid, 89.
Regeneration in relation to healing processes, 546.
Regional differences in antigens of Tubularia, 319.
Release of neurosecretory materials in cockroach, 275.
Reproduction of Chlamydomonas, 54.
Reproduction in Lingula, 202.
Reproductive activity of birds, in relation to light, 601.
Reproductive cycles of chitons, 81.
Respiration of Busycon odontophore, 504.
Respiration of marine eggs, 133, 443, 454.
Respiration of marine lamellibranchs, 125.
Respiration of sea weed-inhabiting animals, 594.
Respiration of Uca, 163, 582.
Respiratory metabolism of Cynthia silkworm, 482.
Respiratory quotient of small animals, method for determining, 408.
Reticulopodia, protoplasmic movement in, 100.
Retinular cell of planarian, 511.
Retractor mechanism of starfish cardiac stomach, 185.
REUBEN, J. P., F. BERGMANN AND H. GRUNDFEST. Chemical excitation of presynaptic terminals at lobster neuromuscular junctions, 424.
REUBEN, J. P., R. WERMAN AND H. GRUNDFEST. Anomalous current-voltage relation induced by hyperpolarization of lobster muscle fibers, 424.
REUBEN, J. P. *See* F. BERGMANN, 405.
Revision of genus Gnorimosphaeroma, 154.
Rhabdome of planarian eye, 511.
Rhombnathides, respiration of, 594.
Rhythm in Uca, experimental modification of, 404.
Ribonuclease, effect of on development of sea urchin, 434.
Ribonuclease from squid, 393.
Ribonucleic acid as possible arginase inhibitor, 611.
RIEGEL, J. A. A revision in the sphaeromid genus Gnorimosphaeroma on the basis of morphological, physiological and ecological studies of two of its "subspecies," 154.
RINALDI, R. A. *See* T. L. JAHN, 100.
Roach, neurosecretion in, 267, 275.
Roentgen irradiation of castrate rats, 68.
Role of light in photoperiodic responses of birds, 601.
ROSENBLUTH, R. *See* T. HAYASHI, 396.
ROTH, J. S., AND E. FIORENTINO. The effect of various metabolites on x-irradiated Tetrahymena, 398.
ROTH, J. S. *See* M. EDMONDS, 393.
ROTHSTEIN, H. The effect of sulfhydryl compounds on the colloid state of the cytoplasm of Arbacia eggs, 425.
RUSTAD, R. C. Further observations relating radiation-induced mitotic delay to centriole damage, 437.
RUSTAD, R. C. The inhibition of mitosis in the sea urchin egg by acridine orange, 437.

SAKAI, T. *See* D. M. CONWAY, 409, 410.
Salinity, effect of on morphology of isopod, 154.
SANBORN, R. C., J. A. HASKELL AND F. M. FISHER, JR. Cells of Limulus in tissue culture, 399.
SANBORN, R. C. *See* P. J. MAVRIDIS, 397.
Sand crab, larval development of, 356.

SANDEEN, M. I., AND M. FINGERMAN. Studies by filter paper electrophoresis of the hormones controlling color changes in the shrimp, Crangon, 425.
SANDEEN, M. I. *See* M. FINGERMAN, 412.
SCHOTTÉ, O. E., AND C. B. SMITH. Wound healing processes in amputated mouse digits, 546.
SCHUEL, H. The effect of urethan on the mitotic apparatus of the Chaetopterus egg, 399.
SCHUH, J. E. *See* SR. F. M. SCOTT, 426.
SCOTT, SR. F. M., AND J. E. SCHUH. Intraspecific reaggregations in tunicates labelled with tritiated thymidine, 426.
SCOTT, G. T. Melanophore dispersing action of ataraxic drugs, 400.
Sea-bat, cardiac stomach of, 185.
Sea urchin, embryology of, 492.
Sea urchin development, effects of temperature on, 150.
Sea urchin egg, fertilizin haptens in, 63.
Sea urchin eggs, oxidation of CO by, 443, 454.
Sea urchin embryos, phosphate accumulation by, 133.
Sea weed-inhabiting animals, respiration of, 594.
Seminal vesicle of x-irradiated castrate rat, 68.
SENFT, A. W. Electron microscope study of the integument, flame cells and gut of the human parasite, Schistosoma, 387.
Serological studies on sea urchin egg, 63.
Serology of Tubularia, 319.
Setting of Venus larvae, 308.
Sex organs of castrate rats treated with x-rays, 68.
Sexual activity of Lingula, 202.
Shape of Venus larvae grown at different temperatures, 308.
Shark egg capsules, histochemistry of, 298.
SHAW, W. N. *See* D. E. PHILPOTT, 423.
Shearing forces, role of in protoplasmic movement in Allogromia, 100.
SHIRAISHI, K. *See* L. PROVASOLI, 347.
Shrimp, brine, axenic cultivation of, 347.
SILKOVSKIS, I. *See* P. F. NACE, 420.
Silkworm, respiratory metabolism of, 482.
SIMPSON, J. W., K. ALLEN AND J. AWAPARA. Free amino acids in some aquatic invertebrates, 371.
Size of Venus larvae grown at different temperatures, 308.
Sizes of nuclei in Rana mesonephroi, 208.
Skeleton formation in corals, 239.
SKOGLUND, C. R. Neuromuscular mechanisms of sound production in Opsanus, 438.
Slug, as food for Orthodemus, 119.
SMITH, C. B. *See* O. E. SCHOTTÉ, 546.
SMYTH, T., JR. Paper chromatography of pericardial organs of crayfish, 426.
Snail, myoglobin in odontophore of, 385, 504.
Sodium content of amphibian eggs, 468.
Sound production in Opsanus, 438.
Spawning of chitons, 81.
SPEIDEL, C. C., AND R. H. CHENEY. Relation of ultraviolet-ray dosage to modifications in fertilization and early development of Arbacia, 427.
SPEIDEL, C. C., AND G. G. HOLZ, JR. Motion pictures of mating behavior of Tetrahymena rendered amicronucleate by x-ray treatments, 384.
SPEIDEL, C. C. *See* R. H. CHENEY, 408.
Sperm, Mytilus, ATP-ase of, 327.
Sperm nucleus in fertilization of Allocentrotus egg, 150.
Sperm of Spisula, respiration and motility in, 433.
"Sphere-cells" of Orthodemus, 119.
SPIEGEL, M., AND E. S. SPIEGEL. The extractable proteins of sea urchin embryos, 427.
SPINDEL, W. *See* P. R. GROSS, 395.
Spinning behavior of Cynthia silkworm, 482.
Spisula egg, vital staining of, 518.
Sporocyst of Asymphylodora, 562.
Starfish, cardiac stomach of, 185.
Starvation, effects of on oxygen consumption of Uca, 163, 582.
Stem antigens in Tubularia, 319.
STEVENS, C. F. The magnitude of random threshold fluctuations in the squid giant axon, 427.
Stomach, cardiac, of Patiria, 185.
Streptomycin, use of in culturing clam larvae, 258.
Stress, role of in neurosecretion of roach, 275.
Strongylocentrotus, accumulation of phosphate by embryos of, 133.
Strongylocentrotus eggs, fertilizin haptens in, 63.
Strongylocentrous eggs, oxidation of CO by, 443, 454.
Strontium, uptake of by phytoplankton, 384.
Studies on mechanism of phosphate accumulation by sea urchin embryos, 133.
Studies on tropical and temperate zone Uca, 163, 582.
STUNKARD, H. W. The morphology and life-history of the digenetic trematode Asymphylodora, 562.
STUNKARD, H. W. Progenesis in digenetic trematodes and its possible significance in the development of present life-histories, 400.

Substrate-enzyme relations in chick embryos, 611.
SUBEN, E. *See* P. PERSON, 398.
SUDAK, F. N., C. L. CLAFF, J. R. BLUMSTEIN AND F. J. TEYAN. Chemically induced hypothermia in the rat: the effect of oxazolium salts compared with 2,4-D, 428.
SUDAK, F. N., C. L. CLAFF, F. J. TEYAN AND J. R. Blumstein. Muscular activity in albino rats treated with 2,4-D, 428.
SUDAK, F. N., C. L. CLAFF, F. J. TEYAN AND J. R. BLUMSTEIN. Thermal gradients in rats treated with 2,4-D, 429.
SUDAK, F. N. *See* C. L. CLAFF, 408, 409; F. J. TEYAN, 429.
Sulfhydryl reagents, effects of on Mytilus sperm, 327.
Surf-clam egg, vital staining of, 518.
SWAMI, K. S. *See* W. L. WILSON, 389.
Swiftlet, cave, acoustic orientation in, 497.
Synapses, fine structure of, 390.
Systematics of isopod, 154.
SZENT-GYÖRGYI, A. G. *See* W. H. JOHNSON, 382; I. ISENBERG, 397.

TANNING in shark egg capsules, 298.
Taurine in invertebrate tissues, 371.
Taxonomy of Chlamydomonas, 54.
Taxonomy of digenetic trematodes, 562.
Taxonomy of sphaeromids, 154.
TAYLOR, F. H. *See* J. T. DAVIES, 222.
Teleost, marine, distribution of diuretic in tissues of, 251.
Teleost, thyroid function in, 89.
Temperate zone fiddler crabs, metabolism of, 163, 582.
Temperature, effect of on metabolism of Uca, 163, 582.
Temperature, effects of on development of Allocentrotus, 150.
Temperature, effects of on enzyme activity of Mytilus sperm extracts, 327.
Temperature, role of in determining size and shape of Venus larvae, 308.
Temperature, role of in respiration of sea weed-inhabiting animals, 594.
TEORELL, T. On the significance of "voltage clamp" data in the "membrane oscillator," 401.
Testis of x-irradiated castrate rats, 68.
Testosterone propionate, effects of on x-irradiated castrate rats, 68.
Tetrahymena, mating behavior of, 384.
Tetrahymena, x-irradiated, effect of metabolites on, 398.
TEYAN, F. J., F. N. SUDAK AND C. L. CLAFF. Oxygen consumption in Uca before and after eyestalk removal, 429.
TEYAN, F. J. *See* C. L. CLAFF, 408, 409; F. N. SUDAK, 428, 429.
Thermal gradients in rats treated with 2,4-D, 429.
Thoracic ganglia of Blaberus, neurosecretory cells in, 267.
Threshold fluctuations in squid giant axon, 427.
Thyroid function in Fundulus, 89.
Tissue culture of Limulus, 399.
Tissues of chick, arginase activity of, 611.
Tissues of marine fish, distribution of diuretic in, 251.
Toad, osmotic studies on eggs of, 458, 468.
Toadfish, distribution of diuretic in tissues of, 251.
Toluidine blue staining of Spisula egg, 518.
Toxin, starfish, effect of on amebocytes, 403.
Trematode, life-history of, 562.
Trematodes, digenetic, progenesis in, 400.
Trevelyana, food of, 439.
Triclad, nutrition of, 119.
Tropical Uca, metabolism of, 163, 582.
TSUBOI, K. K., AND T. HAYASHI. Nucleotide interactions associated with muscle actin transformation, 401.
Tubularia, antigenic differences between stem and hydranth in, 319.
TUCKER, J. S. *See* A. C. GIESE, 81.
Tunicate, vascular budding in, 340.
Turbellarian, nutrition of, 119.
TWEEDELL, K. S. Induced fluorescence in marine eggs, 429.
TYLER, A. *See* R. E. BLACK, 443, 454.
Tyrosinase in Cynthia silkworm, 482.

UCA, metabolism of, 163, 582.
Uca, rhythms in running activity of, 404.
Ultraviolet irradiation of Arbacia eggs, 427.
Unfertilized sea urchin egg, fertilizin haptens in, 63.
Urea formation in chick embryos, 611.
Urechis eggs, oxidation of CO by, 443.

VARIATION between tropical and temperate zone Uca, 163, 582.
Vascular budding in Botrylloides, 340.
Velocity of enzyme reactions in Mytilus sperm extracts, 327.
Venus larvae, destruction of, by bacteria, 258.
Venus larvae, size and shape of, 308.
VERNBERG, F. J. Studies on the physiological variation between tropical and temperate zone fiddler crabs of the genus Uca. II., 163; III., 582.
Vibrio, destruction of Venus larvae by, 258.
DU VIGNEAUD, M. *See* R. L. GREIF, 251.

DE VILLAFRANCA, G. W., AND D. E. PHILPOTT. Fine structure of skeletal muscle from Limulus, 387.
VINCENT, W. S., AND E. BALTHUS. The incorporation of radioactive label into RNA: synthesis or terminal addition, 388.
Vital staining of Spisula egg, 518.
Vitamin C, role of in oxidation of CO by marine eggs, 454.
Vitelline membrane behavior, 410, 411.
Vitelline membrane elevation in Chaetopterus eggs, 284.
"Voltage clamp" data and "membrane oscillator," 401.
Volume changes in amphibian eggs, 458, 468.

WATANABE, H. *See* H. OKA, 340.
Water content of amphibian eggs, 468.
Water relations in injury to Nitella cells, 422.
WATT, W. D. B. The effect of bacteria on radiophosphorus uptake of Fucus, 430.
WEBB, H. M., F. A. BROWN, JR. AND W. J. BRETT. Effects of imposed electrostatic field on rate of locomotion in Ilyanassa, 430.
WEBB, H. M., F. A. BROWN, JR. AND W. J. BRETT. Fluctuations in rate of locomotion in Ilyanassa, 431.
WEBB, H. M. *See* W. J. BRETT, 405; F. A. BROWN, JR., 405, 406.
WERMAN, R., AND H. GRUNDFEST. Graded and all-or-none electrically excitable responses of lobster muscle fibers, 431.
WERMAN, R., AND H. GRUNDFEST. Mechanism of Ba action on arthropod muscle fibers, 432.
WERMAN, R. *See* J. P. REUBEN, 424.
WERNER, G. *See* A. S. KUPERMAN, 390.
West Indian corals, skeleton formation in, 239.
West Indian fiddler crabs, metabolism of, 163, 582.
WHITELEY, A. H. *See* J. B. LITCHFIELD, 133.
WHITING, P. W. Polyploidy in Mormoniella, 402.
WICHTERMAN, R. Action of x-rays on Paramecium: survival and reproduction, 432.
WIESER, W. Life-cycle and metabolism of a marine nematode, Enoplus, 383.
WIESER, W., AND J. KANWISHER. Respiration and anaerobic survival in some sea weed-inhabiting invertebrates, 594.
WILSON, R. S. *See* B. M. JONES, 482.
WILSON, W. L., AND K. S. SWAMI. Electrophoretic studies on protoplasm, 389.
WITTENBERG, J. B. Oxygen transport—a new function proposed for myoglobin, 402.
WOLFSON, A. Role of light in the progressive phase of the photoperiodic responses of migratory birds, 601.
Wound healing in mice, 546.
WRIGHT, P. A. Responses of the skate, Raja, to hyperglycemic agents, 433.

X-IRRADIATED Spisula eggs, dehydrogenase activity of, 415.
X-irradiation of Arbacia eggs, 408.
X-irradiation of Paramecium, 432.
X-ray effects on castrate rats, 68.

YIP, C. *See* P. F. NACE, 420.

ZABLOW, L. *See* R. GUTTMAN, 414.
Zoeae of Emerita, 356.
Zooxanthella, role of in skeletal formation of corals, 239.

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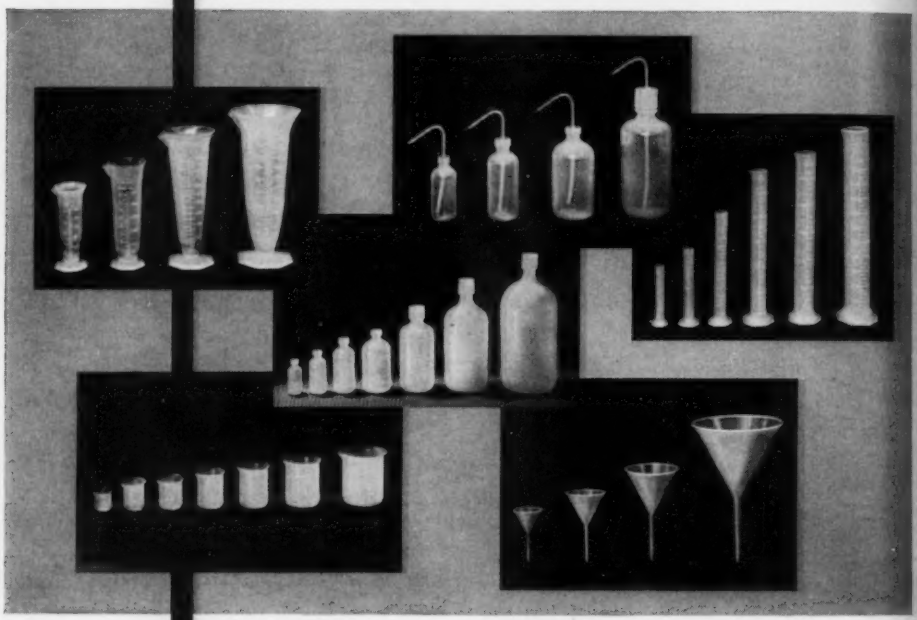

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CONTENTS

Page

ABOUL-ELA, IBRAHIM AHMED
On the food of nudibranchs 439

BLACK, ROBERT E., AND ALBERT TYLER
Effects of fertilization and development on the oxidation of carbon monoxide by eggs of Strongylocentrotus and Urechis as determined by use of C^{13} 443

BLACK, ROBERT E., AND ALBERT TYLER
Cytochrome oxidase and oxidation of CO in eggs of the sea urchin Strongylocentrotus purpuratus 454

DE LUQUE, ORLANDO, AND F. R. HUNTER
Osmotic studies of amphibian eggs. I. Preliminary survey of volume changes 458

HUNTER, F. R., AND ORLANDO DE LUQUE
Osmotic studies of amphibian eggs. II. Ovarian eggs 468

JONES, BRYN M., AND RONALD S. WILSON
Studies on the action of phenylthiourea on the respiratory metabolism and spinning behaviour of the Cynthia silkworm 482

MOORE, A. R.
On the embryonic development of the sea urchin Allocentrotus fragilis 492

NOVICK, ALVIN
Acoustic orientation in the cave swiftlet 497

PERSON, PHILIP, JAMES W. LASH AND ALBERT FINE
On the presence of myoglobin and cytochrome oxidase in the cartilaginous odontophore of the marine snail, Busycon 504

PRESS, NEWTOL
Electron microscope study of the distal portion of a planarian retinular cell 511

REBHUN, LIONEL I.
Studies of early cleavage in the surf clam, Spisula solidissima, using methylene blue and toluidine blue as vital stains 518

SCHOTTÉ, OSCAR E., AND CHARLES B. SMITH
Wound healing processes in amputated mouse digits 546

STUNKARD, HORACE W.
The morphology and life-history of the digenetic trematode, Asymphylodora amnicolae n. sp.; possible significance of progenesis for the phylogeny of the Digenea 562

VERNBERG, F. JOHN
Studies on the physiological variation between tropical and temperate zone fiddler crabs of the genus Uca. III. The influence of temperature acclimation on oxygen consumption of whole organisms 582

WIESER, WOLFGANG, AND JOHN KANWISHER
Respiration and anaerobic survival in some sea weed-inhabiting invertebrates 594

WOLFSON, ALBERT
Role of light in the progressive phase of the photoperiodic responses of migratory birds 601

CESKA, MIROSLAV, AND FISHER, JAMES R.
Arginase inhibition in chick embryos 611